The survival of a Malagasy lemur species

Propithecus verreauxi coquereli

in captivity:

The vital role of a self-selected plant diet

Von der Fakultät für Lebenswissenschaften

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D i s s e r t a t i o n

von Annette Abhau

aus Mülheim an der Ruhr

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For Marcella and Tiberius

«C'est principalement le sens de l'odorat, dont l'étude vient de nous occuper, qui guide les Indrisinés dans le choix de leurs aliments; le goût y est peu de chose, car, le plus souvent, c'est après avoir flairé un fruit ou une feuille qu'ils le rejettent sans l'avoir porté à leur bouche. En captivité, il est fort difficile de nourrir ces animaux; ils refusent presque tous les aliments qu'on leur voit d'abord approcher de leur narines, puis abandonner aussitôt sans y avoir goûté.»

(Milne Edwards A. et A. Grandidier,
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Abbreviations

abbr.	abbreviation, abbreviated
amu	atomic mass units
animal groups:	M&T Marcella and Tiberius D&V Drusilla and Valentinian J&D Julian and Drusilla G Gordian
br.	broad
BSTFA	N,O-bis[trimethylsilyl]trifluoroacetamide
cAMP	cyclic adenosine monophosphate
CI	chemical ionisation
conc.	concentrated
COSY	correlation spectroscopy
d	doublet
DAB	Deutsches Arzneibuch (German Pharmacopoeia)
DAC	Deutscher Arzneimittel Codex (German Drug Codex)
DCI-MS	direct chemical ionisation mass spectrometry
dd	doublet of doublets
DEPT	distortionless enhancement by polarization transfer
dest.	destillata
DGE	Deutsche Gesellschaft für Ernährung (German society for nutrition)
dHex	deoxyhexose
dHex-ol	deoxyhexitol
Diast.	diastereoisomer
DMSO	dimethylsulfoxid
DULC	Duke University Lemur Center, previously known as Duke University Primate Center
EI-MS	electron impact ionisation mass spectrometry
ESI-MS	electrospray ionisation mass spectrometry
FAB-MS	fast atom bombardment mass spectrometry
Gal, gal	galactose
GalA	galacturonic acid
GBF	Gesellschaft für biotechnologische Forschung, Braunschweig
GC	gas chromatography
HAB	Homöopathisches Arzneibuch (Homeopathic Pharmacopoeia)
Hex	hexose (aldohexose)
HexA	hexuronic acid

Hex-ol	hexitol
HMBC	heteronuclear multiple bond correlation
HPLC	high pressure liquid chromatography
hRf	$100 \dfrac{\text{distance between starting line and spot}}{\text{distance between starting line and front}}$
HR-MS	high resolution mass spectrometry
HSQC	heteronuclear single quantum correlation
Hz	Hertz
ID	identification number
IPB	Leibniz-Institut für Pflanzenbiochemie, Halle / Saale
J	values for coupling constants
K-gal-rha	kaempferol-3-O-α-L-rhamnopyranosyl(1→2) ß-D-galactopyranoside
K-rha	kaempferol-3-O-α-L-rhamnopyranoside
LC-MS	liquid chromatography mass spectrometry
m	mass
M	analyt molecule (mass spectrometry)
MHH	Medizinische Hochschule, Hanover
min.	minute
MS	mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)-2,2,2-trifluoroacetamide
MTBSTFA	N-methyl-N-[tert-butyldimethyl-silyl]trifluoroacetamide
MW	molecular weight
n.d.	not detected
NDF	neutral detergent fibre
NHE	natural habitat enclosure
NMR	nuclear magnetic resonance
No., no.	number
ÖAB	Österreichisches Arzneibuch (Austrian Pharmacopoeia)
o.l.	older (autumn) leaves
org.	organic
p.a.	pro analysis
PAS	periodic acid-Schiff reagent
Ph.Eur.	Pharmacopoeia Europaea
Ph.Helv.	Pharmacopoeia Helvetica
prob.	probability
q	quartet
Q-gal-rha	quercetin-3-O-α-L-rhamnopyranosyl(1→2)ß-D-galactopyranoside
q.s.	(lat.) quantum satis
QZ	“Quellungszahl”, swelling number
r_s	regression coefficient
RDA	retro-Diels-Alder reaction
Ref.	reference

ret.	retention
Rha, rha	rhamnose
resp.	respectively
rpm	rotations per minute
s	singlet
sh	shoulder
t	triplet
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TMS	tetramethylsilan
trace am.	trace amounts
u	units
USP	United States Pharmacopoeia
v	volume
v.br.	very broad
veg.	vegetables
WHO	World Health Organization (Report on Infectious Diseases, 1999 & 2000)
xyl	xylose
y.l.	younger (autumn) leaves

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A General Introduction

1 Subject of the study

Propithecus verreauxi coquereli, the Coquerel's sifaka, is a lemur species of the indriid family, which inhabits the northwest region of Madagascar: a mixed deciduous and evergreen forest. It is a diurnal, arboreal living primate. The genus *Propithecus* is one of the most widely distributed among lemurs, but is currently listed as "endangered" by the IUCN Red List (Mittermeier *et al.,* 1994) because of severe habitat destruction (Richard, 1978a; Klopfer and Boskoff, 1979; Harcourt and Thornback, 1990; Ganzhorn and Abraham, 1991).

The digestive tract of *Propithecus* species is highly specialised, requiring a mainly folivorous diet, supplemented with fruits, flowers and to a lesser extent, other plant parts (Richard, 1977; 1978a, b; 1979).

Although many efforts have been made to introduce members of the indriid family into captivity (Spelman *et al.*, 1989), response of indriid species (and subspecies) has been varied. Until recently, it has been difficult to recognise and to fulfil all dietary needs when held in captivity and only a few successful breeding colonies exist outside Madagascar. Changes and imbalances in the diet are often answered by gastrointestinal disorders with diarrhoea as a prominent, recurrent symptom. Diet has an essential role in captive management and it is the key question as to what constitutes a healthy diet (Haring, 1988; Spelman *et al.*, 1989; Harcourt and Thornback, 1990; Powzyk, 1997; Campbell *et al.*, 1999; Campbell *et al.*, 2000).

The only successful breeding colony of captive *P. v. coquereli* is kept at the Duke University Lemur Center (DULC) in North Carolina, USA (previously known as Duke University Primate Center).

Prior to this study animals were given the chance to select leaf species on their own which led to surprising effects: animals improved in health and increased activity pattern while increasing infant survivalship (Haring, 1988). In this study I will ascertain the full dietary needs of captive Coquerel's sifakas. As health conditions have improved significantly with the availability of leaves as a large portion of the daily food before the onset of this study, its benefit for the sifakas will be surveyed. Hence, both the primary and secondary plant compounds will be analysed to assess both the nutritional value of the leaves, and the role of secondary plant compounds as integral ingredients of plant tissue and presumably crucial aspect to leaf choice of the sifakas.

2 Systematic and geographic distribution

Originally, the order Primates had been subdivided into the suborders Prosimiae, including the infraorders Lemuriformes, Lorisiformes and Tarsiiformes, and Simiae comprising Old World monkeys, New World monkeys and humans (Sussman, 1979). This classification was based extensively on evolutionary theory, while the prevailing view now divides the order Primates into the suborders Strepsirhini and Haplorhini with respect to morphology (e.g. rhinarium type).

The suborder Strepsirhini (moist, naked rhinarium) comprises the infraorders Lemuriformes (lemurs) and Lorisiformes (loris and galagos), while Tarsiiformes (tarsiers) shifted to the suborder Haplorhini, which also includes the infraorder Simiiformes containing the subinfra-orders Platyrrhini (New World primates) and Catarrhini (Old World primates) including apes, and humans (Hominidae), (Petter and Petter-Rousseaux, 1979; Grzimek, 1988).

About 60 million years ago, the prosimians were the earliest primates to appear in the fossil record. All living Lemuriformes are endemic to the Island of Madagascar although they were subsequently introduced to the Comores Islands by humans (Sussman, 1979). The Lemuriformes are divided into 5 families, including 14 genera with 32 species (Mittermeier *et al.,* 1994). Recent discoveries of new lemur species (Richard and Dewar, 1991), together with the elevation of several subspecies to species status (Mayor *et al.*, 2004) have increased the tally of extant species on the island. Those prosimians living outside Madagascar have continued to embrace a nocturnal lifestyle while among the Lemuriformes there are numerous species that maintain diurnal active periods (Sussman, 1979).

The superfamily Lemuroidea comprises five families, including Indriidae with three extant genera: *Indri*, *Propithecus* and *Avahi*. *Avahi* is the smallest indriid and is nocturnal, while members of the two larger genera *Indri* and *Propithecus* are diurnal.

Recently, the genus *Propithecus* has been controversially discussed with concepts differing in numbers of species and subspecies (Harcourt and Thornback, 1990; Petter and Petter-Rousseaux, 1979; Tattersall, 1982; 1986; Grzimek, 1988; Mittermeier *et al.*, 1994; Mayor *et al.*, 2004). According to Mittermeier *et al.* (1994), *Propithecus* was once considered to consist of three species, *P. verreauxi* (Grandidier in 1867), *P. diadema* (Bennett in 1832) and *P. tattersalli* (Simons in 1988), with *P. verreauxi* and *P. diadema* containing four subspecies each. *P. d. holomenas* was subsumed into *P. d. edwardsi* (Tattersall, 1986). In a recent survey, some subspecies of *P. verreauxi* and *P. diadema* elevated to species status (Mayor *et al.*, 2004), (see Tab. A.1).

Tab. A.1: Species and subspecies of the genus *Propithecus* according to Mittermeier *et al.* (1994) and in brackets with changed species status according to Mayor *et al.* (2004).

P. verreauxi		***P. diadema***		***P. tattersalli***
P. v. verreauxi	(*P. verreauxi*)	*P. d. candidus*	(*P. candidus*)	
P. v. coquereli	(*P. coquereli*)	*P. d. diadema*	(*P. diadema*)	
P. v. coronatus		*P. d. edwardsi*	(*P. edwardsi*)	
P. v. deckeni		*P. d. perrieri*	(*P. perrieri*)	

Following the eastern-western split of most Malagasy flora and fauna with *P. verreauxi* spp. which inhabit the drier seasonal western forests, and the *P. diadema* spp. which inhabit the eastern rainforests (compare Fig. A.1), which ultimately has had a dramatic impact on the availability of plant species as food plants, especially food choice. Therefore regarding the link between regionality-food plant availability, and to prevent confusion with previous studies, I will retain the species concept of Mittermeier *et al.* (1994) in this study.

To have a closer look on habitat conditions and distribution of *Propithecus* species in Madagascar (Fig. A.1), it is necessary to keep in mind climatic conditions and zones of vegetation. Madagascar can be divided into four main climatic regions: the western, eastern and southern part, and the central plateau. The western part is mainly covered by dry woodland-savannah that changes into a mixed deciduous and evergreen forest in the north and into an arid cactuslike Didiereaceae forest and scrub / bush in the extreme south. The eastern part is covered by wet rainforest and the largely deforested central plateau has a temperate climate (Kuhn, 1971; Richard, 1978a; Hladik, 1980; Richard and Dewar, 1991).

While *P. verreauxi* subspecies are found along the western border with *P. v. coquereli* in the northwest part, the larger *P. diadema* inhabit the wet rainforest along the eastern border. *P. tattersalli* is confined to a restricted area in the northeast of the island. *P. verreauxi* is therefore one of the most widely distributed lemur species (Richard, 1978a; Klopfer and Boskoff, 1979), albeit highly endangered.

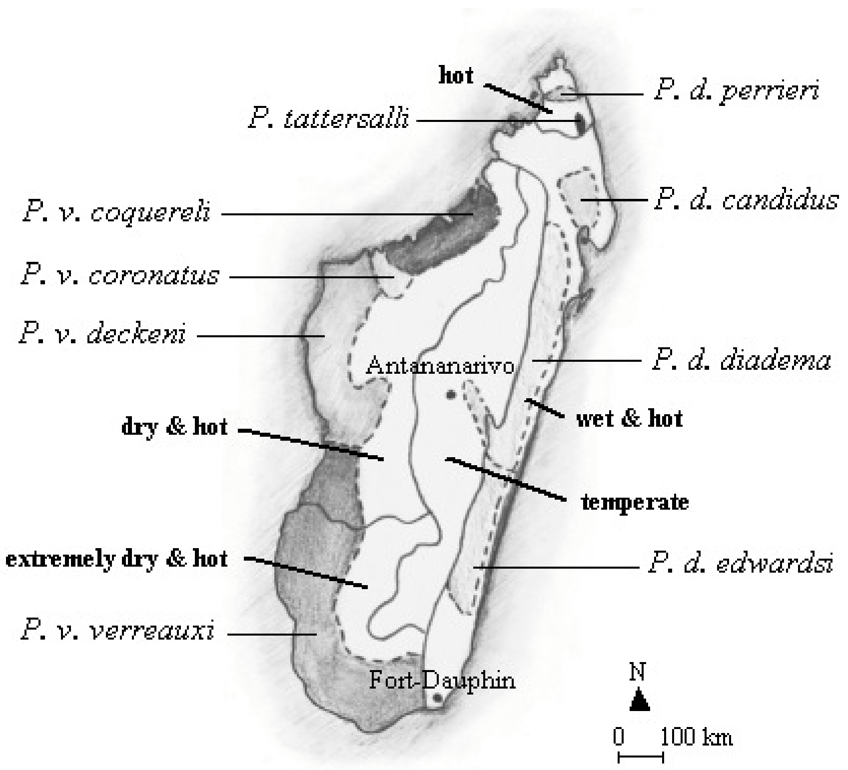


Fig. A.1: Island of Madagascar: Climatic regions (Därr and Wodtcke, 1991) and current distribution of *Propithecus* species and subspecies (Powzyk, 1997).

The term "sifaka" is a Malagasy word and is used for *Propithecus* species in general with special respect to *P. verreauxi*, which refers to their phonetic representation of an alarm call for ground predators. In *sensu stricto* "sifaka" is only appropriate for *Propithecus* species of the dry forest, *P. verreauxi* and *P. tattersalli*.

In contrast, the common Malagasy name for *P. diadema* is "simpona" (Tattersall, 1982), who give a different vocalisation (i.e. "sisk") in the same context compared to *P. verreauxi* (Petter and Charles-Dominique, 1979; Powzyk, 1997).

Native people have attributed medicinal knowledge to the sifakas, e.g. how to heal wounds by means of the application of leaves (Milne Edwards and Grandidier, 1875), and they were held sacred, owing to their sun bathing behaviour in the morning especially after a cold night (Grzimek, 1988). And for many Malagasy people it is still "fady" (tabu) to hunt and kill indriid species.

3 History and response to captivity

Early detailed reports on indriid species go back to the 18th and 19th century. In 1781, Sonnerat brought one *Avahi* and one *Indri* to France, first as a gift to the Jardin du Roi, and later exhibited in the Museum National D'Histoire Naturelle de Paris. In 1832, the genus *Propithecus* was scientifically named by Bennett, while Grandidier named *Propithecus verreauxi* in 1867. The subspecies *Propithecus verreauxi coquereli*, the Coquerel's sifaka was named by Milne Edwards in 1867, and an era of zoological study commenced (Milne Edwards and Grandidier, 1875).

Even in that early stage of research it was acknowledged that diet was a critical point in captive management for members of the indriid family (ibid.) and it took an additional century for researchers to finally succeed in captive maintenance, at least with some *Propithecus* species. The statements of Jolly (1980) that "you will not see a sifaka in zoos of Europe or America, only in the wild or the National Park of Tananarive" and that "captive sifaka simply look down and never look up again" are fortunately no longer valid for at least some of the indriid species / subspecies.

Currently, there are three successful breeding colonies of *Propithecus* species / subspecies outside of Madagascar: a large colony of *P. v. coquereli* and a smaller colony of *Propithecus tattersalli* at the Duke University Lemur Center in North Carolina, USA, and a large colony of *P. v. coronatus* at the Vincennes Zoo in Paris, France. However, other members of the indriid family are much more difficult to maintain. Neither of the two subspecies of *Avahi*, nor any *Indri indri* have ever been kept for longer than a few months in captivity, not even in Madagascar when caged (Harcourt and Thornback, 1990; Powzyk, 1997). It is most likely that diet plays a key role as seen when captive *Avahi* quickly succumb to ill health and death predicated by bouts of diarrhoea (Ganzhorn and Razanahoera-Rakotomalala, 1985; Harcourt, 1991; Ganzhorn, pers. comm.). Although most lemur species suffer from diarrhoea whenever their diets are altered or inappropriate (Bresnahan *et al.*, 1984; Brockman *et al.*, 1988; Ganzhorn, pers. comm.), indriid species are highly problematic in captivity. Wet forest *Propithecus* species consume a poorer food quality and are more difficult to maintain in captivity than the smaller dry forest species of western Madagascar (Powzyk, pers. comm.). In 1995, two of the three wild caught *P. d. diadema* died at the DULC of organ calcification due to an overload of calcium. Subtle differences within the gastrointestinal tract, even among closely related species, may be reflected in different dietary needs (Campbell *et al.*, 2000). Despite all relationships among the indriids, digestion and digestibility remains a species-specific feature (Meyer *et al.*, 1993). For *Indri indri* known as the most folivorous species within the indriid clade, differences in the digestive tract are clearly documented (Milne Edwards and Grandidier, 1875; Hill, 1953), which correspond to a relatively "poorer diet" preference, rich in leaves, when compared to *Propithecus*' digestive tract which is best suited for a "richer diet" composed of more fruit seeds, whole fruits and flowers together with leaves (Powzyk, 1997; Britt *et al.*, 2002).

P. verreauxi appear to have the least problems with captive management, as they are sometimes kept by the Malagasy on a diet of various fruits (Ganzhorn, pers. comm.). Nevertheless, there were two large colonies of *P. v. coronatus* and *P. v. coquereli* that had been maintained outside Madagascar. Although *P. v. verreauxi* is not currently maintained in captivity, one principal breeding pair lived for years at the DULC. This pair failed to produce any surviving offspring which may have been a result of inbreeding (Harcourt and Thornback, 1990). The effects of captivity can be difficult to document, especially when individuals of the breeding colonies are given on loan to other institutions. Overall, the different response of *Propithecus* species to captive conditions remains unclear (Campbell *et al.*, 1999; Campbell, pers. comm.).

In previous studies on free-ranging *Propithecus* species, various aspects of their social behaviour and feeding ecology have been investigated; for reviews see Richard (1978a), Pollock (1979), Harcourt and Thornback (1990), Powzyk (1997), and Powzyk and Mowry (2003).

In 1970/71 and 1974, Alison Richard focused on free-ranging *P. v. coquereli* in the forestry reserve at Ampijoroa in the north and *P. v. verreauxi* near Hazafotsy in the south of Madagascar (Richard, 1974; 1977; 1978a, b; 1979). Her field observations and analysis were used throughout this study to compare captive Coquerel's sifakas to their free-ranging relatives. The majority of behavioural studies were conducted on *P. v. verreauxi* in the south of Madagascar (Jolly, 1972; Jolly *et al.*, 1982; Richard *et al.*, 1991; Richard, 1985; Richard *et al.*, 1993). Only few studies on *Propithecus* species have been performed in captivity (Haring, 1988; Lopes Santini, 1992; Campbell *et al.*, 1999; 2000, 2001, 2004a and 2004b), yet the two subspecies *P. v. coquereli* and *P. v. verreauxi* resemble each other more morphologically and behaviourally than either of the remaining subspecies (Richard, 1978a).

Previous experiences at the Duke Lemur Center

Many efforts have been made to overcome various difficulties arising from captive management, which resulted in a successful breeding colony of *P. v. coquereli* at the DULC. In 1985, a management program was instigated that changed and improved the diet of *Propithecus*, in particular to overcome the dramatic weight loss and decrease in activity patterns that coincided with the winter season (Haring, 1988).

After foregoing and encouraging introduction of other five lemur species to the North Carolina forest at the DULC (Glander and Rabin, 1983; Glander, 1986), the sifakas received a variety of browse, which was partly based on the leaf choice taken by the other lemur species already being held in forested outdoor enclosures. Different housing conditions have been developed in which the lemurs may travel from their large indoor cages to forested outdoor enclosure which more closely resembles their wild habitat (Haring, 1988). Over a period of two years, different leaf species have been introduced systematically into the diet of the sifakas. To prevent the seasonal crisis of animal's condition in winter, some selected species were frozen in the autumn and fed throughout the winter months. Although non-leaf food items changed throughout the years, leaf species chosen by the sifakas remained nearly the same. Animals who had a history of gastrointestinal disorders with diarrhoea as prominent and recurrent symptom in their anamnesis were doing far better, especially during the winter season when food was supplemented by special leaf species. Against initial reservations, no symptoms of self-poisoning occurred, which has already been evidenced for the five other lemur species housed in forested areas, which were all captive-born (Glander, 1986).

3.1 Classification according to gut and dietary needs

Multifactorial interrelations exist between the gastrointestinal tract of an organism and its diet (Parra, 1978; Savage, 1986; Stevens, 1988; Meyer *et al.*, 1993). On the one hand, mammalian herbivores can be classified as forestomach- or midgut-fermenters[1] due to their respective digestive system which requires a special food quality (Langer, 1986; 1988; Chivers, 1994). On the other hand, an organism can be classified according to the food ingested regardless its digestive strategy (Chivers and Hladik, 1980). Frugivores and frugivores / folivores are less

[1] The former term "hindgut" as site of caeco-colic fermentation is revised by Chivers (1994) as "midgut" based on embryological origin. According to Chivers (1994), "hindgut" refers only to the descending (left) colon and rectum, which are very similar in all mammals, and have a different blood supply from the caecum and right colon. The caecum and right colon develop embryologically from the "midgut" loop (Chivers, 1994). So, in this study, the previously utilised term "hindgut" is replaced by "midgut".

specialised regarding their respective digestive tract and diet than species which are predominantly folivorous.

Except for colobines, which have a voluminous sacculated stomach (Ohwaki *et al.*, 1974; Langer, 1988; Stevens, 1988), most primates are midgut-fermenters (caecum and right colon), more or less specialised (Langer, 1986; 1988; Stevens, 1988; Chivers, 1994). Seen from the dietary aspect, most primates can be classified as frugivores that supplement their diet either with leaves or animal matter (Chivers and Hladik, 1980). Depending on the classification nomenclature, herbivorous species which also prey on animal matter can be called omnivores (Langer, 1986) or faunivores / frugivores (Chivers and Hladik, 1980) based on the dominant food preference. According to the latter classification, the digestive tract of frugivores is intermediate between the two extremes: faunivores and folivores. In this study the term herbivore is only used in *sensu stricto*, that is animals which subsist exclusively on plant tissue. Therefore, digestive strategy may be in opposition to a species' respective feeding strategy or feeding ecology, the latter emphasising the inclusion of additional adaptations of an herbivore to its environment.

However, climate, soil, seasonal availability of food and food quality are integral environmental determinants resulting in numerous specialisations as well as convergent adaptations which have evolved over millions of years (Richard and Dewar, 1991). Finally, both, availability of appropriate food and the functioning of the digestive system predispose an organism to occupy an ecological niche (Langer, 1986; 1988).

3.2 Digestive adaptations

In general, digestion is dependent on dentition, and in turn on the gastrointestinal tract of the animal. This includes its salivary glands and saliva composition, the enzymatic equipment of the digestive tract and associated organs, digestive fluids, pH of different sections of the gastrointestinal tract, the microflora of the gut where fermentation of plant material and especially of cell wall structures takes place. In addition, the morphology of the gut system itself (e.g. length of any part, volume, surface area and structure in relation to body weight and size) is also of vital importance (Chivers and Hladik, 1980; Langer 1986; Savage, 1986; Stevens, 1988; Hofmann, 1991). Parameters such as retention time and gut passage rate influence the nutritional exploitation of the food ingested but is vice versa influenced by the food and the microflora of the gut (Warner, 1981; Hofmann, 1983; Marcus and Heaton, 1986; Stevens, 1988; Meyer *et al.*, 1993; Wischusen *et al.*, 1994).

A fibre rich, leafy diet needs more time to be fermented than a more frugivorous diet with less portions of fibre. Therefore, the mean retention time of the gastrointestinal tract is larger for folivores than for frugivores. An appropriate capacity for food storage and fermenting chambers are needed either in the forestomach and / or in the midgut. Hence, numerous investigations on the digestive tract and diet focus on the relationships between body size, digestive system morphology, and overall diet (Chivers and Hladik, 1980; Warner, 1981; Martin *et al.*, 1985; Demment and Van Soest, 1985; Stevens, 1988; Maisels, 1993; Bruorton and Perrin, 1991; Ford and Davis, 1992).

Other mutual and multifactorial relationships exist between a host's microflora, the mucosa and the diet ingested. The indigenous microflora both affects and is affected by the rate of digesta passage (which slows down e.g. in germfree animals), as well as the turnover of and enzymatic activity in enterocytes of the host and the mucosal surface. The enterohepatic circle would not function without the enzymatic impact of the microflora catalysing multiple chemical reactions. While the microflora is essential for the deliverance of vitamins, both the microflora and enterocytes of the mucus layer are engaged in various absorptive processes and provide nutrients and energy for the animal host (Savage, 1986; Stevens, 1988).

Diarrhoea is a cardinal symptom of gastrointestinal disorders and the influence of the indigenous microflora on the absorption of water and electrolytes is noteworthy.

Food composition and environmental factors in turn influence the different microbial communities of the mammalian digestive tract in number and species composition, and their respective microbial activity (Wolin, 1981; Ehle *et al.*, 1982; Savage, 1986; Stevens, 1988; Meyer *et al.*, 1993; Onderdonk, 1998). Factors such as age and overall health of the animal host, greatly influence the microbial ecosystem as well as the living conditions: the microbial colonisation differs enormously between humans living in developing countries and those living in developed countries (Savage, 1986). Microbial enzyme systems are inducible and can vary significantly in different human populations (Ehle *et al.*, 1982). This is the highly pertinent as microbial colonisation does not start until birth (Hoogkamp-Korstanje *et al.*, 1979; Stevens, 1988; Onderdonk, 1998). Likewise, digestive enzymes of host animal tissues are inducible and levels of enzymatic activity may vary with environmental and host factors (e.g. animal species, diet, stage of an animal's development and health condition) (Stevens, 1988; Meyer *et al.*, 1993; Onderdonk, 1998).

There are essentially three sets of factors responsible for determining types and numbers of organisms present within the large intestine. These include: (1) host factors such as anatomy, secretions into the gastrointestinal tract, peristalsis and local immune response; (2) dietary factors including both exogenous and endogenous components; and (3) bacterial-related factors including adhesins, bacteriocins, cofactors, synergistic relationships, antagonistic relationships and commensal relationships (Onderdonk, 1998). In the following analysis, only those factors relevant for this study will be discussed.

Both the proportions and the morphology of the gut may change either for individuals or for the entire species throughout evolution when and if environmental and dietary factors change. For instance, dietary changes have already been evidenced to cause changes in gut proportions of primate species, even after a short time of captivity (Chivers and Hladik, 1980). In response to different food qualities, especially fibre contents between seasons (e.g. winter versus summer), drastic adaptations of the digestive tract have been found in ruminant species (Hofmann, 1983). Egyptian mummies were found to have a considerably larger caecum than present-day man, which is construed together with morphological features (i.e. sacculated colon), that the archaic Egyptian gut resembles that of a herbivore rather than a carnivore type (Stevens, 1988).

Hence, a balanced microbial flora is essential for a normal functioning of the gastrointestinal tract and is of great importance for the health of the entire animal host. Herbivores especially rely on their microflora in order to break down the cell wall structures while gaining nutrients from a diet high in plant fibre and low in readily available carbohydrates, (high) quality protein and vitamins (Stevens, 1988). Therefore the digestion by microbes occurs on both cell wall structures as well as on readily available food components which escaped digestion in the upper digestive tract. As a result, soluble carbohydrates, starch, proteins and lipids are digested by gut microorganisms, whether or not the animal has intestinal digestive enzymes that attack these compounds (Wolin, 1981; Gray, 1984; Mendeloff, 1984; Plaut, 1984).

Important carbohydrate sources such as sugars, starch and structural carbohydrates like cellulose, hemicellulose and pectins are fermented to short-chain organic acids (= volatile fatty acids), principally acetic, propionic, and butyric acids, and the gases hydrogen, methane and carbon dioxide. Although microbial fermentation is inefficient for soluble carbohydrates and starch, it is the only means to digest cell wall structures like cellulose, hemicellulose and pectins. Moreover, degradation of plant cell walls makes cell contents more readily accessible. Furthermore, endogenous non-protein nitrogenous sources such as urea, sloughed-off intestinal epithelium cells and mucopolysaccharides as well as non-protein nitrogen and low quality protein from food are digested and upgraded by microorganisms to yield high quality protein. This in turn serves as an important source of protein for the animal (Wolin, 1981; Stevens, 1988; Langer, 1988). The benefits of caecotrophy and coprophagy in order to gain and preserve nutrients (Stevens, 1988) will not be elaborated on further within this analysis.

However, regardless of the exogenous or endogenous origins of carbohydrates, the production of short-chain fatty acids are important sources of carbon and energy (Savage, 1986; Stevens, 1988). The presence of short-chain fatty acids in the midgut of carnivores, omnivores, and herbivorous mammals suggests that microbial fermentation may be ubiquitous to the midgut (hindgut) of all terrestrial vertebrates (Stevens, 1988). Therefore, the type and amount of dietary fibre has an important impact on composition and quantity of short-chain fatty acids produced (Wolin, 1981; Ehle *et al.*, 1982; Hofmann, 1983; Mendeloff, 1984; Stevens, 1988; Cook and Sellin, 1998; Onderdonk, 1998; Campbell *et al.*, 2002). Although microbial digestion of dietary components is not essential for humans, production of short-chain fatty acids by microbial degradation of various carbohydrate sources largely occurs in the human colon (Wolin, 1981; Ehle *et al.*, 1982; Mendeloff, 1984; Demling, 1995; Cook and Sellin, 1998). In spite of a controversial discussion with respect to whether there is energy gain for human host cells from short-chain fatty acids, their beneficial effects on bacterial growth and on the equilibrium of the microbial ecosystem as well as on various intestinal functions are indisputable (Wolin, 1981; Mendeloff, 1984; Stevens, 1988; Cook and Sellin, 1998; Onderdonk, 1998). For instance some short-chain fatty acids inhibit the growth of several bacterial pathogens that can cause intestinal infections (Wolin, 1981). In case of the human's digestive tract, fibres with gelling polysaccharides greatly influence gut motility and interact largely with absorptive and secretory processes, and influence e.g. blood glucose and cholesterol levels. Furthermore, different fibre types as well as their fermentation products are discussed in the prevention of various gastrointestinal disorders and diseases such as colon cancer (Wolin, 1981; Ehle *et al.*, 1982; Mendeloff, 1984; Plaut, 1984; Cook and Sellin, 1998).

Therefore, forestomach-fermenting mammals with or without rumination as well as midgut-fermenting herbivores with microbial fermentation either in the caecum or colon or both, house an enormous number of bacteria (and some protozoa) with fermenting properties capable of yielding essential nutrients and energy sources.

A well-functioning symbiosis between microflora and their host is indispensable when it comes to the health and welfare of the animal host as a whole (Savage, 1986; Onderdonk, 1998). In that symbiosis, the mucosal barrier of the gut plays an important and multilateral role. On one hand, the mucus layer and the microflora influence each other, on the other hand they build an important barrier against harmful chemical substances and a barrier to colonisation and prevent bacteria and viruses from penetrating into the host. The immune system of the mucosa has distinct structural and functional features as compared to the systemic immune system, and represents in many respects a separate immunological entity (Marth and Zeitz, 1998). So, the gut microflora holds protective functions either by stimulation of immune mechanisms, or by direct competition with pathogenic organisms (Stevens, 1988; Gabe *et al.*, 1998; Marth and Zeitz, 1998; Meddings *et al.*, 2003).

3.3 The phenomenon of diarrhoea

All influences that are disruptive to the microbial ecosystem within the gastrointestinal tract, or the equilibrium between microflora and host, often lead to disorders that are either confined to the digestive tract or include the entire organism.

Reasons for possible disturbances are numerous and often multifactorial. They reach from exogenous factors such as changes in food composition, invasion of detrimental bacteria and viruses, toxic substances, and antibacterial drugs, to endogenous factors such as auto-immune diseases and toxic metabolites that are produced by the indigenous microflora. Overall, these factors result in gastrointestinal disorders with diarrhoea being the most striking symptom. An animal experiencing an acute bout of diarrhoea dehydration is a life threatening condition risk, a more chronic affection of absorptive processes leads to malabsorption and nutrient deficiencies, wasting syndrome and persistent weight loss. Moreover, if the mucosal barrier is severely affected by infections, an inflammatory bowel disease or cancer may cause a dysfunction or degeneration. However, these diseases are more general and impact the overall immune system of the entire animal rather than a disease which is only confined to the digestive tract.

3.4 Implications of morphology

All indriid species are well adapted to an arboreal life. They can be described as vertical clingers and leapers of medium to large body size (Richard, 1978a, b; Powzyk, 1997). Adult *P. v. coquereli* weigh approximately 4 kg (Tattersall, 1982). All indriids are midgut-fermenting primates consuming a predominantly folivorous diet. Within the Indriidae family, *Indri indri* shows the highest degree of specialisations in regard to folivory (Powzyk, 1997; Powzyk and Mowry, 2003).

One of the first detailed description on indriid morphology was given in 1875 by Grandidier (Milne Edwards and Grandidier, 1875). Both lemurs and lorises show a unique feature in their dental morphology. The toothcomb of the lower jaw incorporates incisors and canines which allow for scraping and stripping techniques utilised in the collection of plant tissues such as gum exudates, bark and leaves. Although gum feeding has not been reported for indriids, the common ancestor of lemurs and lorises may have subsisted on gum to some extent (Charles-Dominique, 1977; Petter and Petter-Rousseaux, 1979; Martin, 1979). However, the Indriidae have only four teeth instead of the usual six in their lower toothcomb (Milne Edwards and Grandidier, 1875; Petter and Petter-Rousseaux, 1979; Maier, 1984). The shape of teeth differ for *Indri* and *Propithecus*: *Indri's* teeth are even more adapted to shearing leaf material (Martin, 1979; Maier, 1984; Powzyk, 1997).

The entire digestive tract of *P. v. coquereli* is highly specialised. Indriids possess hyper-trophied salivary glands, voluminous stomachs, sacculated ceaca and looped colons (Milne Edwards and Grandidier, 1875; Hill, 1953; Powzyk, 1997). A recent work of Campbell *et al.* (2000) on *P. v. coquereli* and the related species *P. tattersalli* revealed a spiralled colon and a sacculated caecum, possessing three distinct teniae running the entire length of the caecum, with no sacculations present in the colon. Both the colon and the caecum are embedded by an extensive network of blood vessels. Milne Edwards and Grandidier (1875) previously described the extensive arterial blood vessels embedding the gastrointestinal tract. Although the *Propithecus* and *Avahi*, genera exhibit these aforementioned specialisations, it is most pronounced in the *Indri*. These physiological consequences together with a (possibly) reduced metabolic rate may be in evidence for many of the arboreal folivores including the indriids as well as other prosimian species (McNab, 1978; Müller, 1983; Richard and Dewar, 1991; Ross, 1992; Lopes Santini, 1992) and need further investigation.

The ratio of total intestine length (small intestine + caecum + colon) to body length was found to be 13.0 : 1 for *P. tattersalli* and 15.9 : 1 for *P. v. coquereli* (Campbell *et al.*, 2000). According to measurements by Milne Edwards and Grandidier (1875), the ratio of intestine length to body length is 14.7 : 1 for *P. diadema*, 17.9 : 1 for *Indri*, and 18.9 : 1 for *Avahi* with differing proportions of small intestine length, caecum length and colon length as related to body length between species. However, ratios within indriids are much greater than any other lemur species of a non-indriid family that consumes a more frugivorous diet (Campbell *et al.*, 2000). For *P. tattersalli* and *P. v. coquereli*, the sacculated caecum is presumed to be the principle site for microbial fermentation of plant cell wall components resulting in the production of short-chain fatty acids (Campbell *et al.*, 2000). However, it is striking that in *P. v. coquereli* the length of the small intestine is nearly the same as measured for the colonic section, which is about 3.5 m respectively (Campbell *et al.*, 2000). This obviously pleads for the importance of a well functioning colonic ecosystem to contribute to food processing and to maintain a well functioning gastrointestinal system.

Gut passage rates are in the same range found in other folivores, such as howler monkeys. For both *P. v. coquereli* and *P. tattersalli*, a mean transit time of 17-19 hours with a mean retention time of 31-34 hours was observed under experimental conditions at the DULC (Campbell *et al.*,1999). Another feeding study on *P. v. coquereli*, with experimental diets revealed a transit time of about 23-26 hours with a mean retention time of 30-38 hours (Campbell *et al.*, 2004b). In a study using different sized radio-opaque barium-impregnated polyethylene spheres to characterise gastrointestinal transit times, the animal needed about 10 hours until the first small spheres left the stomach while it took nearly 36 hours for the large spheres to depart. The focal animals needed 24 hours in total for the first small spheres to appear in the rectum. The long storage time in the stomach, contributes significantly to the entire transit time and reflects the species-specific manner of food processing by *P. v. coquereli* (Campbell *et al.*, 2004a).

P. v. coronatus living at the Zoological Park of Paris exhibited a mean retention time of about 23 hours under experimental conditions (Lopes Santini, 1992). In comparison, for humans a mean transit time of about 37 hours was reported (Clemens and Phillips, 1980).

The olfactory sense is well developed within prosimian species and evidenced by scent marking behaviour and the presence of scent glands. *P. v. coquereli* males differ from females in their possession of a throat gland. For a discussion of pheromones see Schilling (1979). As scent-marking is an important feature for communication, social behaviour and ranging pattern, it can be presumed that smell and taste are also important prerequisites for diet selection, as evidenced by feeding trials with *Lemur fulvus* (Glander and Rabin, 1983).

Hence, the difficulties in maintaining animals of the indriid family in captivity seem to be closely related to their highly specialised digestive system which requires a specialised diet. As already mentioned, even among closely related species, the digestive system of an animal including its associated organs, will dictate the digestibility of its preferred diet.

4 Plant secondary metabolism and self-medication

Secondary plant compounds are widely distributed among the plant kingdom and serve to fulfil numerous functions, which are not initially apparent. While some compounds attract insects and even primates as pollinators (Gautier-Hion and Maisels, 1994) or as seed dispersers (Garber and Kitron, 1997), most chemical compounds function as a type of defence, especially as feeding deterrents against insects. Although plant eating insects represent the most important selective pressure on plants, this has led to a coevolution between the two, yet other compounds may have evolved as a means to resist plant pathogens such as fungi, bacteria and / or viruses. The concept that plant secondary compounds are just waste products has long been discarded (Glander, 1982). From the viewpoint of a plant, its specific array of secondary compounds – which often interfere with or function as primary compounds – have evolved to cope with its surrounding environment, its ecosystem including all biotic and abiotic factors.

The way primates, especially folivorous primates, cope with the challenge of secondary plant compounds has yielded a large range of hypotheses and theories, each with a different appreciation of the impact of secondary compounds on primate’s feeding behaviour.

Early approaches evaluated secondary compounds mainly as toxic substances and being energetically-costly as they utilise energy in order to be detoxified. This should result in a heightened avoidance of secondary compounds by herbivores (Schoener, 1971; Freeland and Janzen, 1974; Janzen, 1978; Richard, 1978a). "Consuming plant secondary compounds is a potentially dangerous and metabolically expensive enterprise." ... "Herbivores should therefore prefer to feed on plant parts containing small amounts of secondary compounds" (Freeland and Janzen, 1974). Thus, sensory capabilities and memory are important prerequisites to be able to recognise and avoid toxic substances (Freeland and Janzen, 1974).

Hence, two opposing feeding strategies are thought to have evolved within those animals that consume large volumes of plant foliage. Generalist herbivores are believed to feed on a wide range of different plants as a means to detoxify an array of small doses of secondary plant compounds, while not ingesting a large detrimental dosage of a single toxin. Specialists are thought to concentrate on fewer food resources, and in turn become highly specialised in their ability to detoxify the compound(s) that occur in much higher dosages (Freeland and Janzen, 1974; Glander, 1982; Glander *et al.*, 1989).

Cases have been documented of specialists such as the koala that consumes substantial amounts of essential oils from *Eucalyptus* species (Zoidis and Markowitz, 1992), or the golden bamboo lemur (*Hapalemur aureus*) which is sympatric with two other bamboo lemurs, and is able to consume large amounts of cyanide in its preferred food, emergent tips of the giant bamboo (Glander *et al.*, 1989). Both of these animal species occupy special ecological niches which require special adaptations e.g. as to detoxification systems. Especially for sympatric species, food partitioning and food selectivity are prerequisites that make coexistence possible and current studies are increasingly dedicated to that problem (Charles-Dominique, 1979; Ganzhorn, 1985; 1987; 1988; 1989b; Glander *et al.*, 1989; Richard and Dewar, 1991; Overdorff, 1992; 1993; Powzyk, 1997; Powzyk and Mowry, 2003).

Although the rather rigid paradigm that an animal feeds to maximise nutrients and energy, while minimising its consumption of energy-costly secondary plant compounds has fallen out of favour, it has not been completely abandoned (Glander, pers. comm.). To current opinion, secondary plant compounds influence primate food choice in that they are more likely to be actively avoided than actively sought out (Glander, 1982). However, an animal will exhibit a highly selective diet as a result of the highly variable distribution of primary and secondary plant compounds throughout plant societies and ecosystems. This has been seen in a wide range of Old World and New World primates and prosimian species (Oates *et al.*, 1977; Glander, 1982 (review article); Ganzhorn, 1986; Ganzhorn, 1988; Ganzhorn, 1989a, b; Power and Oftedal, 1996).

"Since plant secondary compounds are ubiquitous, it is highly probable that they influence primate food choice. There is, however, no *a priori* reason to assume this impact is either negative or positive, or that either is mutually exclusive. The challenge lies in determining how the system works" (Glander, 1982).

As already mentioned, there is a close interdependency between diet and gut structure. A healthy animal with good nutrition is closely linked to both a quality and quantity of food appropriate to that animal's gastrointestinal tract and the digestive system. According to Langer (1988) "nutritional niches are clearly related to anatomical differentiations of the gastrointestinal tract". ... "Adaptations of the digestive tract in relation to diet need much more appreciation and coevolution between the digestive tract including associated organs and dietary limitations widen or narrow an ecological niche."

Dentition, digestive fluids, gut morphology, bacterial flora have contributed to the integrity of a single organismal unit, which is the basis for its diet selection and its metabolism (Savage, 1986; Stevens, 1988). There must have been a coevolution between availability of food, e.g. spatial distribution of trees, seasonal availability of leaves, flowers, fruits and other plant parts, and the adaptations to food processing and digestion, as well as abilities and techniques of reaching and handling the desired food items. In short, there are different factors that have influenced the requirements necessary for a specific ecological feeding niche (Hladik, 1978; Garber, 1987; Richard and Dewar, 1991; Gautier-Hion and Maisels, 1994; Powzyk, 1997). Behavioural adaptations as well as the daily activity pattern and / or home range size in relation to diet was the subject of many primatological field studies (Tattersall, 1977; Richard, 1978a; Milton and May, 1976; Milton, 1980; Engqvist and Richard, 1991; Strier, 1992; Powzyk, 1997).

However, the digestive tract is a crucial factor in defining the life-style of an organism. Diet massively influences the well-being of an animal by directly affecting the gastrointestinal tract. Nausea, vomiting, and diarrhoea are essentially uncomfortable disorders supposedly influencing sensory capabilities and learning in regard to food choice (Johns, 1990). Dietary deficiencies become extremely visible, if captively maintained animals fall ill or die. Folivorous primates, as in members of the strepsirhine indriid clade (Spelman *et al.*, 1989), together with Old World primates like the colobines (Collins and Roberts, 1978) and even species with a more frugivorous diet like the New World primate *Saguinus oedipus*, the cotton-top tamarin, succumb in captivity to disorders and diseases concerning the gastrointestinal tract and associated organs. *Saguinus oedipus* easily suffer from ulcerative colitis, a chronic inflammatory bowel disease, and subsequent colon cancer in captivity, that seems to be related to dietary and gastrointestinal factors (Chalifoux and Bronson, 1981; Madara *et al.* 1985; Podolsky *et al.*, 1985; Clapp *et al.*, 1988; Tardif *et al.*, 1988; Watkins *et al.*, 1988; Winter *et al.*, 1989; Targan *et al.*, 1992; Podolsky *et al.*, 1993; Stonerook *et al.*, 1994; Power and Oftedal, 1996; Wood *et al.*, 1998; 2000; Tobi *et al.*, 2000).

Difficulties in husbandry of folivorous herbivores often show disparate success rates in their adjustment to a captive diet and this can be extended to arboreal folivorous mammals such as the folivorous marsupials (e.g. the koala, *Phascolarctos*), the edentates (e.g. sloths, *Bradypus*) as well as the panda (*Ailurus*) (Collins and Roberts, 1978). Adjustment to an appropriate diet in captivity is the prominent challenge for all institutions involved in their maintenance.

Of course, leaf-eating primates rely on primary compounds as carbohydrates, proteins and fat as sources of nutrients and energy as does every animal. There is no doubt that nutrient quality and quantity should be balanced in the diet (Glander, 1982). However, folivores encounter a rather large variety of secondary compounds in different amounts with different pharmacological effects inherent to numerous plant species and parts (Janzen, 1978). The amount of primary and secondary compounds can vary significantly with each plant species, plant parts and climatic season, forcing to a selective feeding behaviour (Fig. A.2). However, the question arises, as to whether the relation between herbivory and secondary compounds is only of unidirectional quality, resulting in minimising and / or avoidance of secondary compounds. Or could it be reasonable to assume, that certain classes of secondary compounds are actively sought out?

The principle of balancing a species primary compound needs should be extended to its secondary compound needs / tolerances with a possible positive effect on the other. Interactions and synergistic effects between and within both groups of primary and secondary compounds and their interference with the digestive system of a herbivore are expected to occur widely (Fig. A.2).

From a pharmacological point of view, primary and secondary compounds are orally administered at the same time, e.g. during one meal. While application and release build the pharmaceutical phase and concern all primary and secondary compounds alike; absorption, distribution, storage, metabolism and elimination processes build the pharmacokinetic phase which also concerns substances occurring naturally in the animal body. Pharmacological effects and side-effects build the pharmacodynamic phase. Extent and rate at which an agent is taken up by the body to be available at the site of action, is called bioavailability. Processes of absorption, distribution, metabolism and elimination occur at the same time, and rate and speed are depending on age, body-size, partly on sex differences, enzymatic equipment and health condition of the animal host (including humans) (Mutschler, 1996). The enzymatic equipment may vary between populations as indicated in the occurrence or absence of the enzyme, lactase, in humans (Gray, 1984). Enzyme systems as well as receptors can be induced or inhibited depending on the agent to be processed.

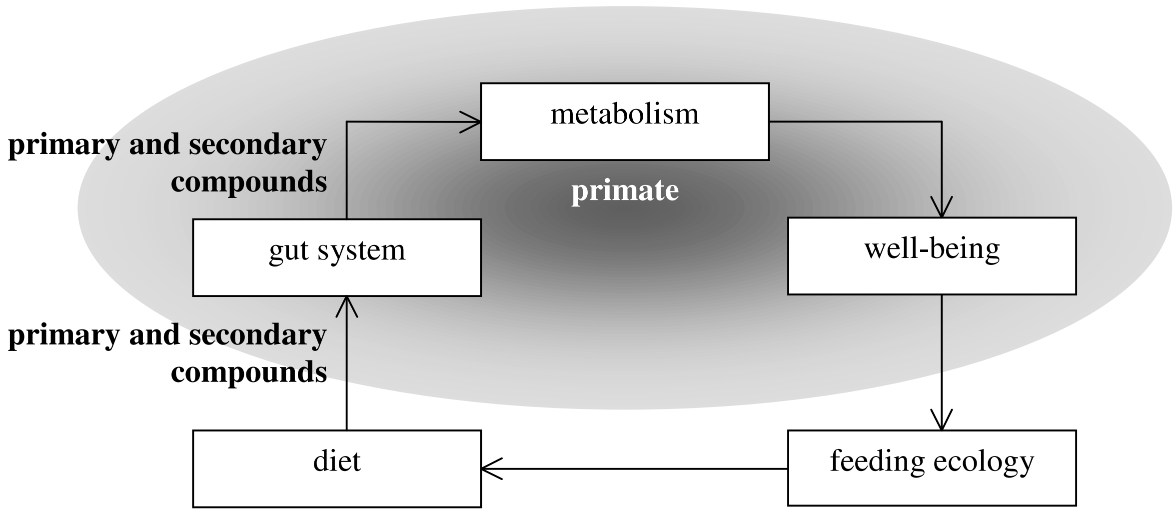


Fig. A.2: Relationship between an organism such as a primate and its environment. Factors influencing the ecological niche are viewed from the standpoint of the primate.

Digestive processes already commence in the mouth by enzymes contained in the saliva, alleviated by both the chewing and grinding of the food. In monogastric mammals like humans, the absorptive processes occur mainly at the surface of the intestinal lumen. Metabolic processes predominantly occur in the liver, but also in the mucosa as well as by the gastrointestinal microflora and intestinal enzymes that are produced by the animal host itself. While in humans, the gastrointestinal microflora plays only a minor role in detoxification processes, yet especially in herbivorous mammals, the impact of gut microbes either in the forestomach, midgut or both are of great importance (Langer, 1988; Stevens, 1988). Moreover, the gut participates in the enterohepatic circle, which can prolong the effects of pharmacologically active compounds which have either been ingested or produced by the body itself.

In this context, one facet of plant use deserves far more appreciation: the use of medicinal plants. It needs to be emphasised that self-medication by means of secondary plant compounds may improve health conditions and life quality, prolong life-span and may ultimately be viewed as evolutionary adaptation. While medicinal plants are well accepted in human medicine for acute or chronic diseases, called phytotherapy, their role for animal species is just beginning to be understood. Extensive research has been done on chimpanzees as our closest relative and how they utilise certain plants for medicinal purposes (Wrangham and Nishida, 1983; Wrangham and Goodall, 1989; Huffman and Seifu, 1989; Dossaji *et al.*, 1989; Mason, 1990; Sears, 1990; Page *et al.*, 1992; Jisaka *et al.*, 1993; Clayton and Wolfe, 1993; Koshimizu *et al.*, 1993; Ohigashi *et al.*, 1994; Huffman, 1995; Huffman *et al.*, 1997; Page *et al.*, 1997). The special way of ingesting plant tissue like swallowing entire leaves by chimpanzees and bonobos (Newton and Nishida, 1990; Huffman *et al.*, 1997; Dupain *et al.*, 2002) has quickly attracted the researcher's attention and ethnobotanical applications in regard to humans have also been examined. Leaf-swallowing is viewed as a form of drug dose, with the intended pharmacological effects delivered to the gastrointestinal tract as in the removal of parasites. Indeed, it seems that primates suffer from numerous gastrointestinal parasites (Ohigashi *et al.*, 1994; Landsoud-Soukate *et al.*, 1995; Stuart and Strier, 1995; Huffman *et al.*, 1997; Dupain *et al.*, 2002). With respect to the strong feeling of nausea in gastrointestinal disorders, it is not surprising that a large range of present day phytopharmaca in human medicine is dedicated to gastrointestinal disorders and associated digestive processes within the gut including organs such as the bile, liver and pancreas. In this respect the bitter taste is obviously an important clue to chimpanzee's phytotherapy (Newton, 1991; Koshimizu *et al.*, 1994).

However, while the application of phytotherapy among primates in case of acute disorders is relatively obvious, long-term use of medicinal plants for chronic disorders or the application of subtherapeutic dosages are far more sophisticated and in turn, difficult to analyse. Recently there is an increased awareness of the impact of secondary compounds in the human's diet, and this awareness results in a voluminous market of supplementary products containing extracts or compounds of various plant species.

As the cognitive and perceptive abilities in primates increased during evolution, this too is highlighted in the development of human medicine and pharmacy. Humans learnt to cope with secondary compounds in plants within their environments, which then led to a selective use of plants for medicinal purposes. Knowing this, it is just a small step to hypothesise that the daily food selection of folivorous primates is more likely to select those secondary plant compounds which exhibit beneficial effects onto their gastrointestinal tract and their well-being rather than just minimise the intake of plant chemicals and subsequent detoxification processes. When substantial amounts of plant tissues are selected and their corresponding high levels of secondary compounds are ingested (e.g. essential oils by the koala, cyanide compounds by *Hapalemur aureus*), other purposes besides the obvious detoxification processes are to be presumed. If secondary plant compounds are conceived as challenge and chance to occupy a novel niche, they should yield an evolutionary advantage and a contribution to the fitness of the herbivorous animal. With respect to the aforementioned adjustment of the digestive tract to diet and vice versa, the secondary compounds should be included in this paradigm and their subsequent impact on niche occupation and health benefits should be thoroughly investigated.

5 Major goals

The major goal of this study is to examine the role of the leaves beneficial to the well-being of *P. v. coquereli*. On one hand, leaves are an important dietary food item for nutrients and energy supply, on the other hand, the behaviour of selective leaf choice pleads for additional factors influencing food composition. The diversity of secondary plant compounds as integral ingredients of any plant tissue, provides numerous ecological functions and pharmacological effects and is therefore predestined to influence the feeding behaviour of herbivores. Based on active leaf choice of captive Coquerel's sifakas and the finding that overall health improved significantly when different leaf species had been introduced into the diet, justify the need to examine the role of the leaves in the sifakas' diet with particular respect to the occurrence of secondary plant compounds and their health benefits.

At the onset of this study, no detailed data have been available on food intake of Coquerel's sifakas in captivity at the DULC. Consequently, qualitative and quantitative aspects of the diet will be surveyed to assess food composition. Different holding conditions including access to forested habitat enclosures of the North Carolinian forest and seasonal changes in leaf composition will be scrutinised in their importance that influence food choice.

To assess the seasonal influence on leaf composition, two different study times were chosen: spring (May) and autumn (October). Observation of feeding behaviour will reveal general and individual food preferences in order to document preferred, less preferred and avoided plant species and to take into account seasonal dependencies. Food intake will be based on weights and measures of food plants as related to body weight of the focal lemurs. Quantitative determinations of primary compounds shall reveal food composition of carbohydrates, fat and proteins and caloric needs with special respect to the impact of the leaves.

To determine the leading secondary plant compounds in the most preferred food plants, preparative analyses with subsequent structure elucidation will be conducted. Quantification of the leading compounds in the diet and subsequent relation to bodyweight shall build the basis for pharmacological dosage comparisons with human medicine. Pharmacological assays with special respect to gastrointestinal disorders will complete the beneficial aspects of the selected secondary plant compounds for the sifakas. Less selected or avoided food plants will be screened for their secondary compounds mainly by literature study to review less beneficial or avoided compounds.

This study design implies several advantages: 1. Holding and feeding conditions do not have to be changed in order to study feeding behaviour, 2. Data collection can start from the first day on. Therefore, no time is needed for the habituation of animals to this experiment. The method of data collection on feeding behaviour within the outdoor enclosures was developed to be applicable to wild field conditions. Two study periods have been chosen, both periods long enough to gain reliable feeding data, and small enough to analyse the contents of primary and secondary plant compounds of the respective food species and parts as constant. In order to determine intended dosage calculations of ingested plant compounds it is advantageous to collect feeding data on consecutive days. Furthermore, changes in contents of primary and / or secondary plant compounds (as predicted for the different seasons) are expected to mirror changes in the feeding behaviour and hence the consumption of the respective plant tissue.

With respect to qualitative plant analyses, methods will be employed that can easily be transferred to the wild (e.g. thin-layer chromatography). This shall encourage other researchers to analyse a focal animal's food plants within their natural habitat. This will give researchers a better understanding of the animal's food choice, and dietary aspects which will serve to improve an animal's overall health when captive.

Hence, two short moments in the life of captive Coquerel's sifakas were chosen to investigate two major problems, which will be treated in Part B and C of this study, respectively:

Part B is dedicated to the observation of the feeding behaviour and quantification of food intake on a fresh weight basis. The investigation of the food choice shall reveal food preferences or avoidance of plant species and parts, which might either be based on individual taste, or overall feeding pattern which may or may not follow seasonal constraints. The basic question is: **What do the animals eat?**

Part C is designed to analyse the main food plants for their contents of primary and secondary plant compounds. Of course, quantification of the secondary compounds necessitates a series of sophisticated analyses for characterisation and structure elucidation of the major components to know what the sifakas actually ate. Assays will be conducted to reveal possible pharmacological effects of the leading secondary plant compounds. Quantification of the major secondary products and calculations of actual dosages ingested by the sifakas will be the basis for this dosage-corresponding relationships and comparisons to human medicine. Hence, the following question will be assessed:

What dietary factors are responsible for the sifakas' well-being?

B Empirical study on food selection

1 Introduction

The following study is designed to monitor the feeding behaviour of captive *P. v. coquereli* during two seasons with respect to dietary composition of food species and the respective quantities eaten. This study was conducted at the Duke University Lemur Center (DULC) in North Carolina, USA. Based on previous studies, active food choice has been well documented in a variety of lemur species (Milne Edwards and Grandidier, 1875; Glander and Rabin, 1983; Glander, 1986; Haring, 1988) and can be used as an important tool to decipher which plant species are worthy of analysis.

A total of six animals were studied during two study periods, the first in the spring of 1994 and the second in the autumn of 1997. Animals were kept in a variety of enclosures, including various combinations of indoor cages, outdoor cages and / or free-ranging forested habitat enclosures. The focal lemurs received daily feedings of a wide variety of fruits and vegetables augmented with commercial leaf-eater primate chow. In addition, a clump of browse was cut from various tree species growing proximate to the DULC forest and were then brought into the animal's cages on a daily basis. Two of the four study groups have had long standing access to forested habitat enclosures. These groups were especially important in observing how *P. v. coquereli* responded to the variety of plant species available under natural conditions.

Observations were conducted during the main feeding times when animals were in their cages and / or in the outdoor forested enclosures. The sifakas were observed to discriminate between different food types as seen in their disparate handling of potential food plants from various trees, shrubs and climbing plants. The recording of a feeding pattern helped to discern which foods were preferred and which were avoided by the focal lemurs. These observations highlighted the species-specific food requirements for *P. v. coquereli* as indicated by their preferred food specimens. A classification system of the different plant species was then recorded to reflect the general behaviour of the animals towards the different plant species and plant parts within their environment and in respect to seasonal differences.

The entire food intake is based on each plant's fresh weight, the parameters of each lemur group, together with the lemurs' kg bodyweight. If no direct weighing of food items was possible (e.g. feeding on wild browse in outdoor enclosures), the corresponding weight of food intake was estimated. Seasonal variations in dietary preferences were taken into account as well as any variations attributable to the needs of animals of disparate ages.

Both, the qualitative and quantitative analyses of the important food constituents with particular respect to those leaves selected by the animals were used to help identify which secondary plant compounds are crucial in food choice and may ultimately bear beneficial effects toward the health of the Coquerel's sifakas. In summary, observations from Part B will establish the basis for subsequent nutritional, chemical and pharmacological investigations on those plant species that prove vital in the maintaining the robust health of the six captive *P. v. coquereli* within the four study groups.

2 Materials and Methods

2.1 Animals

The feeding behaviour of a total of six animals assorted in four groups were studied during two different seasons, spring and autumn (Tab. B.1). Two groups were studied during each period, respectively. The first observation period occurred in spring, lasting from the 5th to 23rd of May in 1994. The follow up study was conducted in the autumn from the 30th of September to the 9th of October in 1997. At periods of observations, the group of Marcella and Tiberius (M&T) and Julian and Drusilla (J&D) consisted of adult[1] animals, respectively. In contrast, the group of Drusilla and Valentinian (D&V) and Gordian (G) consisted of juvenile (Drusilla and Gordian) and subadult (Valentinian) sifakas.

Tab. B.1: The table represents the study animals and how they were grouped during both study seasons. Data of birth and bodyweights are included[a].

name (abbr.)	ID[b]	date of birth	spring '94 weight	spring '94 group	autumn '97 weight	autumn '97 group
Marcella (M)	6110 f	summer 1981[c]	4830 g	group M&T		
Tiberius (T)	6207 m	20. Jan. 1988	3700 g	group M&T		
Valentinian (V)	6450 m	2. Nov. 1991	3400 g	group D&V		
Drusilla (D)	6538 f	6. Apr. 1993	2550 g	group D&V	4470 g[d]	group J&D
Julian (J)	6518 m	4. Mar. 1993			4060 g	group J&D
Gordian (G)	6650 m	17. Dec. 1995			3000 g	group G

a The bodyweights depicted in Tab. B.1 were treated as being constant throughout the short study times. For fluctuations of bodyweights in captivity and in the wild see Glander *et al.* (1992).

b Identification number

c Date of birth was estimated at time of capture; wild caught 11.6.1986.

d As Drusilla gave birth to an infant on 7th of February 1998 with a presumed gestation period of 160-165 days (Campbell, pers. comm.), she was presumed to be pregnant during the observation time in the autumn. Richard (1978a) reported a gestation period in the wild of about 130 days; 162 days are reported by Tattersall (1982).

Three study groups consisted of two animals, one female and one male, respectively. The fourth group (G) comprised only one male. As he had a broken foot, he was kept alone.

Whereas group M&T had partial access to the natural habitat enclosure NHE-1 during the spring, in the autumn group J&D was kept in NHE-3 during the entire study period and spent more hours per day in the forest than group M&T. The other two groups only stayed within indoor cages during the study periods (see Fig. B.1 and Fig. B.2).

When group M&T was observed, it was the third time for Marcella to be outside in a natural habitat enclosure. Tiberius was there for his first time during the observation period in spring 1994. In contrast to group M&T, group J&D had already been well habituated to the forest when the study was started in the autumn 1997. Marcella and Julian have been collared for most of the study time and could therefore easily be identified in the forest. Otherwise,

1 1-2 years: juvenile; 2-3 years: subadult; > 3 years: adult, although growth may continue (Richard, 1978a).

animals within the same group could be identified by their appearance regarding size, weight and physiognomy.

Typically, adult females are reported to be the dominant group members within a group's feeding hierarchy, with the exception to juveniles and subadults being displaced by adult animals (Richard, 1978a; Richard and Dewar, 1991). The same behavioural pattern was found for the groups of this study. In the group of Drusilla and Valentinian, the juvenile Drusilla was chased away several times during a feeding bout by the older Valentinian. Dominance behaviour in this study is only important as far as access to food is concerned. It may be that some aggression was a means to avoid inbreeding (father-daughter) yet studies of their social behaviour lie beyond the scope of this study.

In spring 1994, group M&T was studied for three weeks, group D&V for one week. In the autumn 1997 all study animals were observed for a period of 11 days (Tab. B.2). In May, both study groups were kept during the first week in their indoor cages. For the second week, group M&T moved to a holding cage in NHE-1 to be acclimatised to the surrounding forest and to a group of *Eulemur coronatus* already residing there. During the third week (17.-23.5.1994) the sifakas were allowed to go out into the forest for several hours per day. The daily sojourn terminated abruptly when Marcella vigorously attacked the group of *E. coronatus*.

Tab. B.2: Duration of the study periods and number of days used for data record.

Group	**M&T**	**D&V**	**J&D**	**G**
No. of days of study periods	19[a]	7	11	11
No. of days for data record of leaves	18	7	11	11
No. of days for data record of fruits & vegetables	16	7	11	10

[a] Group M&T was studied for 6 days in their indoor cage, 6 days in the holding cage of NHE-1 and 7 days in the forest of the natural habitat enclosure NHE-1.

Discrepancies between the number of days comprising a study period and the number of days utilised for data collection were due to various intrusions such as when staff interfered with the procedure of this study, e.g. when the study groups had already been fed by the staff before food refusals of the last feeding bout could be collected back, data had to be discarded.

2.2 Feeding conditions

Except for group J&D, all animals were fed twice a day. Food was composed of fruits and vegetables, primate chow, and leaves (Tab. B.3). In the morning, animals received fruits and vegetables including chow; in the evening they received a bunch of leaves, called "browse". Only group J&D received an evening feeding of all types of food after having spent the day outside in the forest.

Fruits & vegetables were offered in feeding bowls, one for each animal, respectively. Browse was cut in the forest nearby and hung up within the cages. Water was given *ad libitum*.

While Marcella and Tiberius got a total of 21 different species of fruits and vegetables and 8 different leaf species during their study time, the other three groups received a total of 20 different species of fruits & vegetables and 6 (group D&V) or 7 (groups J&D and Gordian) different leaf species, respectively (see Tab. B.3). As easily can be seen from this table, plant species offered tended to vary.

Tab. B.3: Food species offered in the cages to the study groups. Figures indicate the frequency, how often a designated food species was given to the respective study group.

fruits & vegetables	group M&T	D&V	J&D	G
apple	12	4	8	6
banana	4	2	2	4
Brazil nut			3	1
broccoli	4	3	1	4
cantaloupe[a]	7	3		
carrot	15	4	4	3
cauliflower	3	2		
celery	1	2	2	3
chick pea	1	3	7	8
chow[b]	16	7	11	10
cucumber	14	3	10	7
French bean			2	
grape[c]	3	2		1
kale[d]	11	3	7	6
kiwi fruit	1	1		1
mango	2	1	1	
muskmelon[a]	3	1		
mustard, green			1	1
onion			1	2
orange	8	2		
pea (pods and seeds),green[e]	3	4		
pear			4	4
plum			1	2
red cabbage			2	2
sweetcorn	1		2	1
sweet potato	13	4	11	10
watermelon	1	3		
white cabbage	2	2	1	1

leaf species	group M&T	D&V	J&D	G
Rhus copallina	18	7	11	11
Albizia julibrissin	18	7	10	10
Robinia pseudoacacia	4	2	2	2
Acer rubrum	17	6		
Cercis canadensis	14	5	2	2
Liquidambar styraciflua	17	6	5	5
Liriodendron tulipifera	1		3	3
Photinia glabra			1	1
Mangifera indica	1			

[a] Both the cantaloup(e) and honeydew melon were fed to the animals, and were available in U.S. markets. These melon types can be interpreted in their vernacular names as muskmelon, *Cucumis melo* L. To indicate that two different sorts were fed, cantaloupe was recorded as cantaloupe and honeydew melon as muskmelon.

[b] Mazuri Leaf-eater Primate Diet, No. 5672, Purina Mills, Inc., St. Louis, MO, quality controlled by PMI Feeds, Inc.

[c] Grape: violet and red sorts were fed.

[d] Kale: frizzy and smooth sorts were fed.

[e] Two varieties of peas were fed.

Rhus copallina (sumac) and *Albizia julibrissin* (mimosa) were the most consistent leaf species fed to the study animals. In 1994, additionally *Acer rubrum* (red maple), *Cercis canadensis* (redbud), and *Liquidambar styraciflua* (sweetgum) represented the bulk of leaves which were offered nearly daily to groups M&T and D&V. In 1997, both study groups typically received three out of seven different leaf species per day, yet received four species on a single day during observation. Normally, *R. copallina* and *A. julibrissin* plus a third leaf species built the bulk of leaves presented to the groups J&D and G. However, regarding the leaf species, both groups of the same study period received (with one exception) the same leaf species on days they were simultaneously studied.

2.3 Holding conditions

Cages

Basically, “indoor” cages were identical equivalents. Boxes with heating lamps were installed to provide heat during cold days. Half of the cages of groups M&T and D&V were covered by a roof. Gordian’s cage was totally covered. (Group J&D was not observed in indoor cages.) Several large trunks provided platforms for jumping and sitting. In the cage of group D&V some bamboo was growing. In each cage, fresh air and UV-light were in abundance.

Holding cages within the natural habitat enclosures were larger, without any roof yet large boxes were provided for shelter. Detailed descriptions are given by Haring (1988).

Natural habitat enclosures

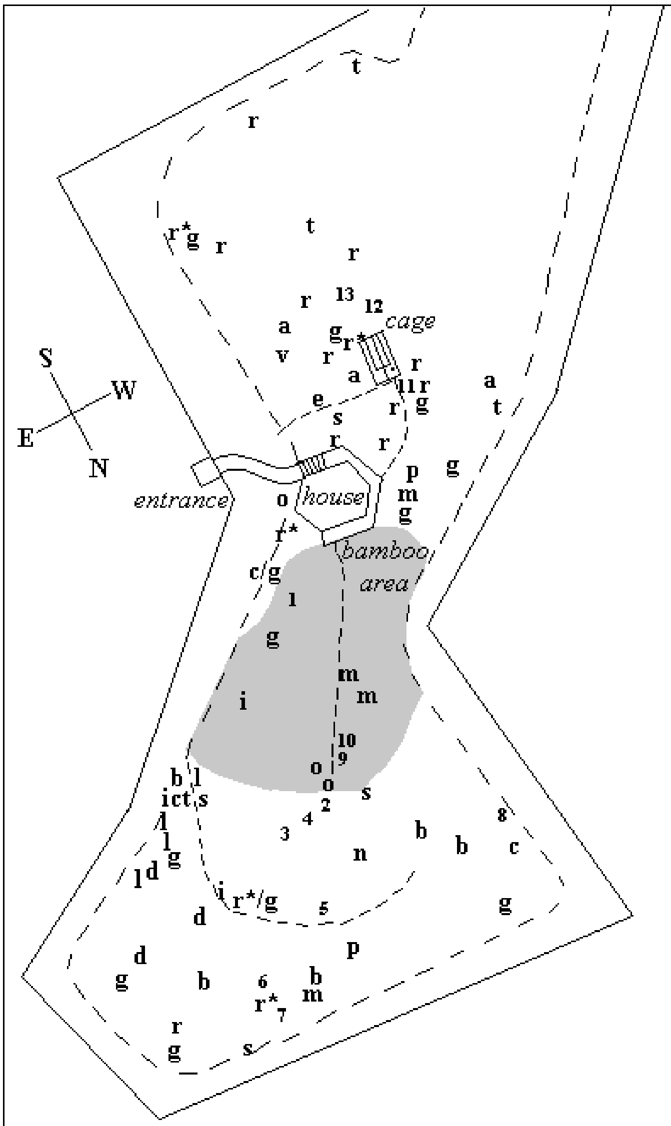
The feeding behaviour of the *Propithecus* groups were undertaken in the two natural habitat enclosures of NHE-1 and NHE-3. During the spring, group M&T was studied in NHE-1 comprising 0.6 ha (Fig. B.1), and during the autumn, group J&D in NHE-3 had a 1.5 ha area (Fig. B.2). All natural habitat enclosures were surrounded by an electric fence. A broad lane of grassland that was several meters in width, prevented the animals from escaping through the trees and jumping over the fence. Within the NHE-1 enclosure, a house was built near the entrance that was used for seminars and was typically closed. It served as preferred sitting and climbing facilities for the focal lemurs. More details are given by Glander (1986) and Haring (1988).

Both, NHE-1 and NHE-3 were mapped out with particular respect to the species of trees, shrubs and climbing plants, from which the animals were observed to eat from, rest in, or jump through. Climbing vines growing on these designated trees were also noted. Additionally, at least one individual tree, shrub or vine of each species growing in the respective NHE was included on the maps as a review of the species diversity within the forested site of the focal animals. Based on observed activity patterns, the lemurs preferred the northern aspect of NHE-1 enclosure while the other group preferred the southern aspect of NHE-3. This mirrored wild type behaviour as reported by Richard (1978a), who noted that lemurs tend to use certain pathways while travelling within their respective territories. Only the core habitats within the enclosures were carefully mapped out, whereas the animals’ tracks were not depicted on the maps (Fig. B.1 and Fig. B.2). Herbs and additional plant species which comprised the ground layer were of minor interest to the feeding sifakas and are not included on the maps, but are discussed specifically when the animals came down to the ground.

Plant species included on the maps were initially identified using the “Audubon Society field guides to North American trees and wildflowers” (Little, 1993; Niering and Olmstead, 1992), with additional verification by Prof. Dr. R. L. Wilbur of the Duke University Botany Department.

The following species were present in the NHE-1 enclosure, but not depicted in the map (Fig. B.1): *Rhus radicans* (poison ivy; ground layer), *Pinus taeda* (loblolly pine), *Juniperus virginiana* (red juniper), *Ailanthus altissima* (tree-of-heaven) and *Lonicera japonica* (honeysuckle). *Pinus taeda* was the tallest and most widespread tree, growing up to 30 m, while most of the other trees had a height of approximately 4 m at the time of study.

Juniperus virginiana was situated within the large bamboo area and was the preferred residential tree of the *Eulemur coronatus* group.



legend

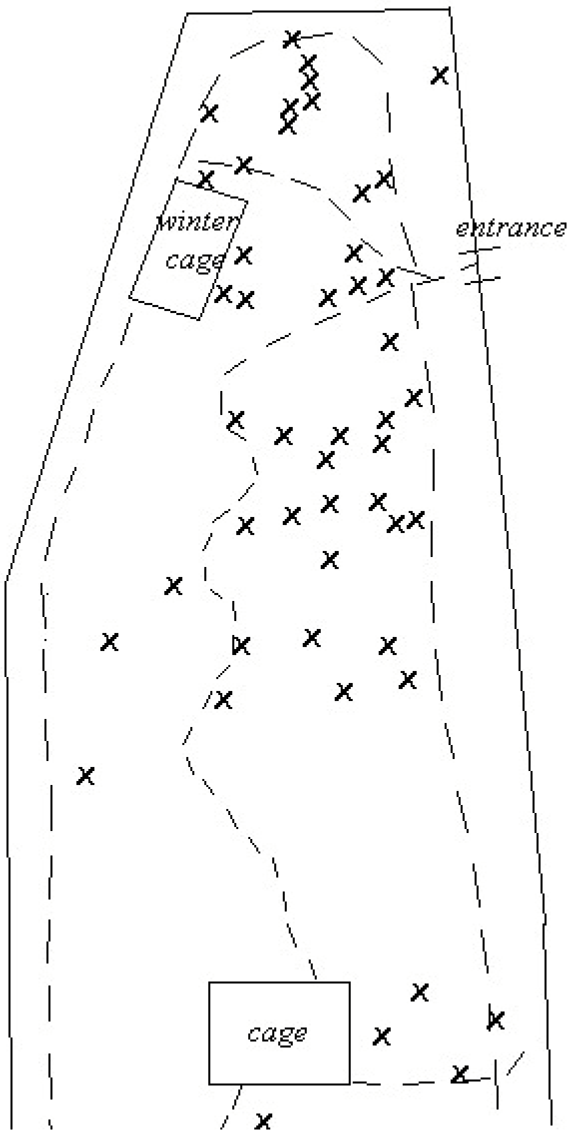
a = *Fraxinus americana* (white ash)
b = *Cercis canadensis* (redbud)
c = *Vitis cinerea* (grape)
d = *Cornus florida* (dogwood)
e = *Ulmus alata* (winged elm)
g = *Liquidambar styraciflua* (sweetgum)
i = *Carpinus caroliniana* (American hornbeam)
l = *Robinia pseudoacacia* (black locust)
m = *Morus rubra* (red mulberry)
n = *Ligustrum vulgare* (common privet)
o = *Quercus stellata* (post oak)
p = *Diospyros virginiana* (common persimmon)
r = *Acer rubrum* (red maple)
r* = *Vitis rotundifolia* (grape)
s = *Quercus falcata* (southern red oak)
t = *Liriodendron tulipifera* (tuliptree)
v = *Viburnum rafinesquianum* (rafinesque viburnum)

1 = bamboo area
2 = *Viburnum rafinesquianum*
3 = *Prunus serotina*
4 = *Quercus phellos*
5 = *Cornus florida*
6 = *Viburnum prunifolium*
7 = *Diospyros virginiana*
8 = *Robinia pseudoacacia*
9 = *Carya tomentosa*
10 = *Fraxinus americana*
11 = *Fraxinus pennsylvania*
12 = *Fraxinus americana*
13 = *Nyssa sylvatica*

— — grass area
- - - - path for better tracking the animals
NHE-1 comprises 0.6 ha.

Fig. B.1: Map of the natural habitat enclosure No. 1 (NHE-1).
Latin and vernacular species names are listed in Tab. B.7. All trees, shrubs and vines that were tasted, eaten or spat out by the sifakas during the time spent in the forest were designated with a letter to mark the species and location. Numbers are used to mark individual trees that were passed by without any contact.

Of those species which were regularly fed as browse in spring 1994, *R. copallina* (sumac) and *A. julibrissin* (mimosa) were not growing within the NHE-1 enclosure (Fig. B.1). The fruit and leaves of *Mangifera indica* (mango) were fed to the lemurs only once in spring to group M&T, and neither was growing outside. Although one tree of *A. julibrissin* was growing within NHE-3 (Fig. B.2), this was of minor importance for this study, as animals had already destroyed the tree and had consumed all available leaves before the onset of this study period. *Photinia glabra* was neither growing within either of the natural habitat enclosures and was presented once as browse during the autumn study period. Another difference between the enclosure NHE-1 and NHE-3 were the *Oxydendrum arboreum* (sourwood) trees, which were found only in NHE-3 and were a preferred food resource for group J&D.



During the follow-up study in the autumn, only the positions of individual trees and species type were marked on the map. Occasionally, the identification was confined to the genus, and this was deemed sufficient. The following genera and species were identified:

Carpinus caroliniana (American hornbeam)
Robinia pseudoacacia (black locust)
Diospyros virginiana (common persimmon)
Cornus florida (dogwood)
Pinus taeda (loblolly pine)
Albizia julibrissin (mimosa)
Carya tomentosa (mockernut hickory)
Quercus sp. (oak)
Juniperus virginiana (red juniper)
Acer rubrum (red maple)
Oxydendrum arboreum (sourwood)
Quercus falcata (southern red oak)
Liquidambar styraciflua (sweetgum)
Liriodendron tulipifera (tuliptree)
Quercus phellos (willow oak)
Viburnum sp.
Vitis sp., mostly *V. cinerea*

— — grass area
\- - - - path for better tracking the animals
NHE-3 comprises 1.5 ha.

Fig. B.2: Map of natural habitat enclosure No. 3 (NHE-3).

2.4 Data collection schedule

Groups M&T, D&V and Gordian were fed twice a day. In the morning, fruits & vegetables and primate chow were brought into the cages for their first feeding. Refusals of fruits, vegetables and chow from the previous day were removed right before the morning session, and refusals of browse from the previous day were removed after that first feeding bout. In the evening, animals received additional leaves with the remaining fruits & vegetables from the morning feed session. This facilitated a high diversity of available foods during the main feeding bouts within the cages. However since the holding cage in NHE-1 with group M&T was not secure from hungry mice and chipmunks who feed on the fruits and vegetables, I had to remove uneaten food (fruits & vegetables) in the evening after the second main feeding bout instead of during the next food session which was during the following morning.

A slightly deviant procedure was performed on group J&D, as they were fed only once a day after having spent the main portion of the day outside in the forest. Of the latter group, all food refusals were collected in the morning of the following day.

All food brought into a cage and collected later was weighed, as food intake was based on fresh weight.

It is worth mentioning that animals who had spent the day in the forest, could be easily lured back from the forest into their holding cages by showing them the bunches of browse. Although many different trees were growing within the forest, animals rapidly followed the browse back into their cage.

Whereas group M&T spent an average of 3 $\pm$ 1 h outside in the forest during one of the three study weeks, group J&D spent 6.5 h outside on a daily basis.

During the study period, group J&D was involved in a movie production (4.10.-7.10.). Therefore they received their browse on these days prior to entering their cage. On 8.10. and 9.10. some students were allowed to observe that group as part of a research project which once again caused a disturbance of the normal activity patterns.

Observational methods

After bringing the food into the cages, the feeding bouts were normally observed for at least one hour due to all breaks made by the animals between feeding on any food item. This method of "focal-animal sampling", is based on Altmann (1974). In this study, one feeding bout is defined as the entire time animals have spent feeding including all short breaks and interruptions for food selection (e.g. for seeking or dropping the food, looking around, chasing each other, or other activities such as jumping, moving, and sunbathing). A feeding bout may commence immediately when the food is brought into the cages, and continues until the last animal to feed has stopped all feeding. The feeding bouts when food is brought into the cages (twice a day for groups M&T, D&V and Gordian; once a day for group J&D) were deemed the most intensive, and were noted as the "main feeding bouts".

This aforementioned feeding bout method differs from other feeding bout methods in that this study all the breaks and interruptions are defined as mere intervals of approximately a minute. The time spent feeding and the estimated amount of food ingested (see estimation method) during this time has been recorded for each individual during the main feeding bouts within the cages and during all time spent within the forested enclosures (App. I.2). In this study, the terms "feeding bout" and "time spent feeding" are adjusted to the requirements of this study and are therefore deviant from common usage as given by Richard (1977; 1978a).

During all main feeding bouts within the cages the first ten (different) food species eaten by each animal were scored, irrespective of how long the animals fed on either food item or what amount was ingested. These sequences are called ranks (numbering from one to ten) and are summed over all observation days, respectively for each animal and separately for the feeding bouts in the morning and in the evening. In App. I.1 feeding bouts with animals eating leaves are presented. Occasionally, when food species were changed less than ten times by a single animal during a feeding bout, that animal had ranks, numbering the first ten different species could not be completed until the tenth rank. Therefore, the sum of frequencies per rank does not always equal the number of days studied per animal. This way, individual food preferences will be discerned from general preferences and seasonal changes. Furthermore, during the main feeding bouts it was recorded what type of food item was selected and estimated as to how much of it was ingested by each individual. Additionally, the method by which the animal treated the food and handled the food was recorded.

Outside in the forested enclosures, focal animals were followed all day. This method is based on “sampling all occurrences...” by Altmann (1974). It was not necessary to define individual feeding bouts for the time outside. Feeding behaviour in the forest was closely observed regarding food preferences and avoidance. Each food item ingested was recorded and converted into corresponding weights. Single events of handling plant species like tasting, eating, spitting out or just jumping through a tree were also recorded to yield a “classification system” of plant species and plant parts reflecting the sifaka’s overall feeding behaviour (Tab. B.7). Those characterisations of plant species, which includes observations within the cages, are needed to evaluate secondary plant compounds inherent to all plant species / parts with which the lemurs make contact.

Quantitative methods to evaluate food intake

Two different methods were applied to quantify food intake: the weighing method and the estimation method. While the weighing method could only be applied to the entire group – it was noted that in the case of Gordian, the weighing method yielded exact information on his individual food intake, and therefore the estimation method could then be applied to each individual and hence completed aspects of individual food intake. Within the forest, the estimation method was the only possible way to quantify food intake. Hence, estimating the amount of food intake within the forest, completed the amount characterised by the weighing method within the cages for those groups who were partly outside and partly fed within the cages.

Weighing method

The daily food intake per group within the cages was determined by the difference of weight between the food brought into the cage and the refusals collected back. Regarding tree species, weight ingested corresponded to the weight of leaves eaten. For subsequent calculations it was necessary that leaves were stripped off the twigs of the remaining bunches that were collected back, whereby the twigs and leaves were weighed separately for each species. To take into account the weight loss of the browse by vaporisation during the time within the cage, a leafy bunch comprising the same leaf species, was stored at equal weather conditions (temperature, wind, shadow, protected from rain) for the same amount of time and treated the same way as the food browse. The daily weight loss by vaporisation was then averaged respectively for each tree species during the study period, and yielded one species-specific factor per season. Hence, for each tree species offered within the cage the amount of leaves eaten was calculated by multiplying browse refusals by the species-specific, seasonal factor of vaporisation and subtracting that value from the weight of plant species originally brought into the cage. To get the amount of mere leaves brought into the cage, the weight of twigs was multiplied by the vaporisation factor which was then subtracted from the weight brought into the cage. While the loss of weight by vaporisation was negligible for fruits and vegetables, the more juicy fruits were ultimately preferred, and were eaten quickly and completely. These calculation were also performed on leafy vegetables such as kale.

Calculation of the weight eaten:

$$weight_{eaten} = weight_{in} - weight_{out} \bullet f$$

$weight_{eaten}$ Weight of food [g] ingested by the sifakas. In case of tree species, the weight eaten corresponds the weight of leaves eaten.

$weight_{in}$ Weight of food [g] put into the cage.

$weight_{out}$ Weight of food refusals [g] collected back from the cage.

f Factor f gives the weight loss by vaporisation, respectively for each leaf species and leafy vegetable per season. Regarding fruits, it was not necessary to calculate such factors.

$$leaves_{in} = weight_{in} - twigs_{out} \bullet f$$

$leaves_{in}$ Quantity of mere leaves brought originally into the cage, which is calculated by difference between the weight of browse brought into a cage and the weight of twigs gained back after the meal while taking into account the loss of weight by vaporisation.

$twigs_{out}$ Weight [g] of the mere twigs got out of the cage; leaves were stripped off.

Estimation method

All food ingested within the cages during the main feeding bouts as well as all plant parts ingested outside in the forest were counted and recorded in characteristics such as size and volume (e.g. number of pieces, bites or mouthfuls of food; number of leaves of a defined size). That way the selected food items were estimated by eye which were then converted into corresponding weights. Therefore, different amounts of food which met the same criteria were weighed several times and then averaged. This method utilised the averaged values on each food species.

This method was used to get the amount eaten by each individual within the cages during the main feeding bouts as well as during the time spent in the forest, where weighing was impractical. This way, the method was validated within the cages by correlating estimated weights to weighed weights and corroborating and complete the “weighing method” by observations of individual food intake.

2.5 Statistics

Due to small data sets, only nonparametric tests were applied (Conover, 1971; Sachs, 1968; Siegel, 1976, Lamprecht, 1992).

The **Spearman-rank-correlation test** (one-sided) was applied to test for a correlation between food eaten and food supplied within the cage with a high correlation expected for preferred food items.

The **Mann-Whitney U-test** (two-sided) was used to test for significant differences between two different plant species eaten by one group, and to test for differences in eating one definite plant species by two of the four groups, respectively. Absolute amounts used are referred to kg body weight of each respective group. Weights of plants eaten in the forest are included in this test. As this statistical test can be utilised on data concerning the entire group, individual preferences are only indirectly tested for.

3 Results

3.1 Qualitative evaluation of the lemur food

The following investigation is dedicated to the treatment and handling of different food species and parts with particular respect to the portion of leaves which were consumed by the focal lemurs.

To evaluate the role of the leaves within the diet, the behaviour was observed in order to record individual differences in the treatment of plants, and in contrast, to recognise any feeding trends exhibited by all study animals. Precise observations shall account for different holding conditions (e.g. cages, outdoor enclosures), different ages of the study animals and different seasons to reveal any possible dependencies. A set of criteria will be established to evaluate the behaviour of food acceptance, clear preference and avoidance towards different tree species offered as browse, and especially any preferences regarding the different species of trees, shrubs and climbing plants available within the forested habitat enclosures (Tab. B.7). This system serves to classify different plant species according to the animal's behaviour and is needed in order to proceed with subsequent investigations on the role of secondary plant compounds and whether they impact food choice (Part C).

Treating and handling the food
An impression of how animals dealt with plant material, handling and processing of the food during the main feeding bouts, needs to be established. This is especially vital when animals were ranging freely in their forested habitat. Apart from fruits and vegetables, the lemurs were generally interested in the leaves of a variety of tree, shrub and climbing plant species. Observations in the forested habitat enclosures revealed, that plant species located on the ground level of the enclosures were of minor interest.

While resting in a tree, a lemur would often scan the forest and decide which type of leaf should be eaten and then move toward the food plant. Depending on whether the branch could carry the weight of the animal(s) and the remaining distance between the lemur and the desired food, leaves could directly be plucked and chewed or a hand could be extended to grasp the twig or stem, and pull the food to the mouth. Whenever they bit off twigs with leaves attached, the lemur would then consume just the leaves (sometimes) including the leafstalk, or only parts of the leaf blade, or the entire leaf. Whole leaves were consumed typically when the leaves were young in age, and depending upon the species of leaf. This involves factors such as leaf age, size, texture, toughness and / or length of the leafstalk. Whenever the lemurs were feeding in an uncomfortable posture, the animals would quickly move back to a more secure feeding position.

To feed on leafstalks, a lemur would gather one to three leaves with leafstalks, which were then bitten off, while the leaf blades were held in their hand. Once the stalks were eaten, the leaf blade(s) was (were) dropped. When just the leaf blades were the preferred food, the leaves were bitten off by their teeth, with just the leafstalk remaining in the hand and subsequently dropped. Except for stalkless species such as *Rhus copallina*, and the very young leaves of a few other plant species, leafstalks were typically not eaten together with the leaf blade: animals either decided in favour of the leafstalk or the leaf blade. For additional information regarding the harvesting and feeding techniques of *P. v. coquereli* and *P. v. verreauxi* in the wild, see Richard (1977, 1978a, 1978b).

Before biting into any food item, the lemurs often sniffed and / or licked the potential food item. That procedure, – look, sniff, lick – was especially performed on novel food specimens or when animals switched from one food species to another. The procedure of deciding which plants are food items has already been described in other lemur species (Glander, 1986; Glander and Rabin, 1983) as well as for members of the indriid clade as in different *Propithecus* species (Milne Edwards and Grandidier, 1875; Powzyk, 1997), which corroborates the inference that food is predominantly chosen by smell and initial taste. Experience translates into knowledge, and these skills as developed by captive-born lemur species which were introduced to forested areas at the DULC (Glander, 1986; Glander and Rabin, 1983) and have never known their indigenous Malagasy diet. For captive-born *Lemur fulvus*, feeding trials revealed that smell and taste were most important in deciding whether a novel plant food could be ingested or not. Vision may play a minor role as one blind animal made the same choices in the same fashion as her sighted mates (Glander and Rabin, 1983). Of course, normal free-ranging (wild caught) primates learn by their parents what to eat (Richard, 1977), but this was not the case for lemur species who were introduced to unknown natural habitat conditions such as the North-Carolinian forest.

With respect to fruits and vegetables, there were some species which were eaten in a particular manner by the study animals (Tab. B.4). In contrast to leaves, all species of fruits and vegetables were ingested in the same manner by all the study animals.

Tab. B.4: Particular way of eating some species of fruits and vegetables.

food species	**remarks on the feeding behaviours of food items**
Brazil nuts	They were processed until shells were crushed. The inner seed was nearly consumed. Animals spent much time processing, crushing and eating nuts.
cantaloupe, cucumber, muskmelon and watermelon	The pericarp and 1 cm of the adjacent pulp were not eaten.
celery	Leaves were preferred.
grape, chick pea and chow	Entire items were eaten, respectively. Grapes and chick peas were the most highly preferred food items and were completely consumed during all observed feeding bouts.
kale and broccoli	Most stalks or pieces with a stalk were not consumed. Leaves of kale and flowers of broccoli were preferentially eaten.
kiwi	Brown peel with some green and yellow fruit bits were not consumed.
orange	Yellow peel (including the albedo-layer) was not consumed. All juicy pulp was consumed in its entirety.
peas (pods and seeds)	Seeds were preferred. Pods that did not contain any seeds were seldom eaten, sometimes only tasted and then dropped.
sweetcorn	Just the inner corn cobs were not consumed.
sweet potato	Typically, only thin pieces of the outer cortex were not consumed.

There were no other specialised feeding behaviours observed in other species of fruits and vegetables that were consumed by the focal lemurs.

With respect to trees, shrubs and climbing plants growing within the forest of the natural habitat enclosures together with the tree branches offered as browse to the animals, the majority of the animals' interest was granted to the leaves. The selection of leaves and plant parts eaten is described in Tab. B.7 together with the behaviour shown towards the various plant species.

Species offered as browse or found in a natural habitat enclosure partly revealed that different lemurs showed disparate food preferences in those animals belonging to the same or to a different group. Intergroup differences might be due to different seasons, which will be discussed for some leaf species.

Observation of the feeding behaviour in the cages

The feeding behaviour of the main feeding bouts within the cages was recorded regarding food species chosen, the first ten different species selected and the corresponding weight which was estimated as being ingested by each animal per food specimen, respectively (B.2.4).

Results showed that the feeding pattern of the different groups was influenced by different cage conditions. When animals stayed exclusively within the indoor cages, fruits and vegetables and chow were consumed occasionally in the evening, and were not among the first ten selected different species, although all types of these aforementioned foods were available. For instance, only once did "Gordian" consume fruits and vegetables in the evening among his first ten different food species, out of all evening feeding observations.

For groups with access to the forest, this behaviour changed markedly from the first day of observation. The sequence of selecting the different sources of food (leaves, and fruits & vegetables and chow) in the evening following an active day in the forested enclosures, differed considerably compared to those lemur groups that resided within inside cages. Therefore it is important to note that group J&D was fed only once a day within the cage and spent more time per day in the forest than group M&T. Animals with outdoor access started to switch to fruits & vegetables during the first ten species right sooner, than those focal animals that resided within the indoor cages (App. I.1).

Marcella sometimes started from rank 5 on, and Tiberius quite regularly by rank 7 to eat fruits & vegetables. Group J&D regularly started with fruits and vegetables including chow on rank 1 or 2, and then switched to leaves of *Rhus copallina* and *Albizia julibrissin* subsequently (App. I.1). It is reasonable to assume that the markedly changed sequence of food selection (food item and plant species) within the first ten ranks may be due to nutrient content such as readily available carbohydrates which have high levels in both sweet potato and chick peas. In fact, a positive correlation between species selected and their contents of soluble carbohydrates and starch, is presumed although this correlation is not statistically significant based on the small sample size. (For the contents of nutrients provided by the different food sources see chapter C.I.) Therefore it is not surprising that one group, J&D started eating fruits and vegetables among their first ten species chosen, earlier than group M&T.

However, it is remarkable that all individuals irrespective of holding conditions showed a strong predilection for both *R. copallina* and *A. julibrissin* leaves within the first ten species during the feeding bouts when browse was available (App. I.1). This is even more remarkable considering that group J&D spent most of the day in the forest foraging, which naturally provides a huge quantity and variety of wild leaves. The preference for these species (*R. copallina* and *A. julibrissin*) emphasises their importance within the sifakas' diet. Furthermore, it is interesting to note that those lemur groups that spent long hours foraging in the forest could easily be lured back into their holding cages by offering them *R. copallina* and *A. julibrissin* browse.

For those animals that were kept exclusively in indoor cages, early morning would typically find them searching over their old browse for any undetected leaves of *R. copallina* and *A. julibrissin* which would have normally been consumed the day before. This again supports the importance of *R. copallina* and *A. julibrissin* leaves within their diet.

The time spent eating during a feeding bout differed widely between individuals and time of day. Groups that were fed twice a day, normally needed additional time in the evening to ingest all desired food items. The highest difference could be found for "Marcella", who spent 34 ± 9 min. feeding in the evening and only 10 ± 5 min. during the morning. Within the group

M&T, Marcella ate less food in the morning than Tiberius, (both in feed time and weight of food consumed), but the converse occurred during the evening feeding. Time spent eating in the morning decreased for Marcella especially when she had access to the forest, whereas time and amount eaten in the evening increased. In addition, Marcella appeared to be more picky than other animals in selecting the food and was observed to drop more food items than Tiberius.

When feeding within the cages, the first ten different species consumed during the main feeding bouts showed a feeding time that typically lasted approximately 10-20 minutes and most food consumed during a feeding bout was eaten within this time frame. The first six to ten different species were eaten very quickly, and than animals commenced to slow down. If the estimated weights of the feeding bouts were compared to the weights eaten that day, it revealed that most of the preferred food items were eaten during the observed time.

Depending on the degree of hunger and the type of food item, it may take different units of time to ingest a food item. If the lemurs switched quickly between food species, the first ten food items were consumed rather quickly but in low quantities. If the focal animals spent a protracted time feeding on any food item, e.g. on *R. copallina*, it was observed that not all the ranks were completed during the feeding bout although a large amount was consumed. It could also occur that much time was spent on a small amount of food, e.g. Brazil nuts, as it necessitates time to pry the nuts away from their shells. In fact, there is no correlation between the amount of food eaten of any food type and the time used to consume that item, which is an enormous dilemma considering that field workers typically measure food intake according to time spent feeding.

Although water was given *ad libitum*, animals observed in the spring 1994 were never seen drinking. On the contrary, animals were often drinking in the autumn 1997. This may be attributed to the water content of the leaves which is normally higher in younger than in mature leaves. Generally, immature leaves in the spring season were more abundant than in the autumn season. Furthermore, food supplies differed largely between the spring and autumn in regard to the quantity of fruits, vegetables and chow offered to the focal lemurs. In the spring, more juicy fruits and vegetables were offered than in the autumn, and in autumn more primate chow was given to the lemurs.

However, the estimated weights of foods consumed during the feeding bouts as well as the first ten different food species chosen support the role of the leaves, especially the preferred leaf species, within the animals' diet. Additionally to the preference of leaf species by all focal animals, individual leaf preferences could be detected. Food items that were eaten in large amounts on a daily basis during the observed feeding bouts by all animals were determined to be more important than those food items only consumed occasionally. Moreover, with respect to leaves, differences in the seasonal feeding behaviour can be clearly distinguished. For species of fruits & vegetables it was not possible to detect any preferences considering the large variety and the fluctuation in food composition.

In the morning, except in the case of "Gordian", two feeding bowls with fruits and vegetables were placed within each cage. Although each animal had "his own" bowl, Marcella typically chased the subordinate "Tiberius" away from "his" bowl, and they exchanged the feeding bowls several times. In the evening, animals displayed aggression regarding the desired food items, e.g. for leaves of *R. copallina* and *A. julibrissin*. Therefore, feeding bouts normally lasted much longer than the feeding time without all the feeding interruptions. Based on age and dominance relationships, especially in the case of "Tiberius" in group M&T and "Drusilla" in group D&V, certain animals had to wait for their feeding opportunities.

Drusilla could feed uninterrupted when "Valentinian" had already eaten the desired amount of his preferred species. If not enough food was provided of a preferred food item e.g. leaves of *R. copallina* or *A. julibrissin*, it was Drusilla that was left without. However, Valentinian was also seen searching the branches of *R. copallina* from which all leaves had been previously eaten. Nevertheless, Drusilla predominantly ate the leaves of *R. copallina* and *A. julibrissin* during the evening feed time as the first ten species when enough food was provided.

"Gordian" was kept alone because of his broken foot, and therefore he had a particular situation. No lemurs chased him away during any feeding bout, but during his feeding activity he was easily interrupted by neighbouring animals, who screamed or made other vocalisations or movements. In his behaviour towards the observer he was very cautious yet curious. Half an hour was the typical observation time per feeding bout which was sufficient to record the food types and amounts that Gordian consumed. Gordian can best be evaluated regarding food intake, as he was alone in his cage. Gordian's feeding pattern resembled that of individuals within the groups during the spring of 1994, who were also fed twice daily.

However, with respect to leaf species available within the cages, a general predilection for *R. copallina* and *A. julibrissin* leaves can be stated for all individual sifakas during the observed main feeding bouts despite different holding conditions, despite particular individual situations, and despite different seasons. In the following, the feeding behaviour towards trees, shrubs and climbing plants growing within the forested enclosures will be examined.

Observation of the feeding behaviour in the forest

Based on the feeding behaviour of group M&T in NHE-1 in the spring and group J&D in NHE-3 in the autumn, all plant species growing in these enclosures, with special respect to the leaves as most abundant plant parts, shall be classified into three major categories:

1.) tasted and eaten as positive reaction,
2.) passed and untouched as sort of indifferent behaviour,
3.) refused as negative reaction.

These major categories shall build the basis for subsequent chemotaxonomical reviews on these plant species and plant parts to assess the pattern of food choice with special respect to the secondary plant compounds (see chapter C.II and App. II.2). In this respect, eaten and refused plants are more important than passed or untouched species. Leading compounds in common within the selected plants and plant parts will then be the basis for subsequent chemical analyses (chapters C.III, IV and V).
In the following, the foraging behaviour of both groups shall be described in detail to result in the "classification system" of plant species according to the aforementioned categories (Tab. B.7; App. II.2). Therefore, animals were followed all the time they had been in the forest.

Group of Marcella and Tiberius

When animals were released into the NHE-1 (map, see Fig. B.1, p. 21), they first passed a tree of *Fraxinus americana* (white ash) standing close to the cage and then climbed to the top of a *Pinus taeda* tree (loblolly pine) (24-30 m), pausing there for approximately ten minutes. *Pinus taeda* trees were the tallest trees growing in the NHE-1. After descending to approximately 4 to 10 m, they started their route, while staying maintaining close contact. The majority of time outside was spent in the central part of their core area of the NHE-1 near the house and their holding cage. Only once were animals observed in the southern part of the NHE-1.

On several occasions, animals came down to sit on the ground. Animals belonging to group M&T were outside for approximately 3 ± 1 hour. Normally, after two to three hours outside, they then moved to the entrance and were resting in the grass lane, as if waiting for food.

Although they rested in the grass on nine occasions, the grass was tasted only three times, in amounts too low to be estimated. Another ground cover in the NHE-1 was *Rhus radicans* (poison ivy). This plant species was never seen to be touched although it was once even stuck into a bunch of browse. Overall, observations were focused on trees, shrubs and climbing plants as the predominant vertical strata used within their forested habitat.

A group of *Eulemur coronatus* also resided within the NHE-1. The large area with bamboo (*Rondumaria tecta*) and a *Juniperus virginiana* tree (red juniper tree) were their main site of residence. The bamboo area was crossed several times daily more or less quickly by Marcella and Tiberius. This *J. virginiana* tree is a vivid example of a plant species that was just "passed" by the focal sifakas, since the majority of encounters with the *E. coronatus* group with Marcella, typically took place near or even in that *J. virginiana* tree. Within this area, only once Tiberius tasted a woody stump and Marcella gnawed at it.

Tab. B.5 reviews the daily feeding behaviour of group M&T toward plant species growing within the natural habitat enclosure NHE-1. Only those plant species that were tasted (less than 5 g were ingested) or eaten (more than 5 g were ingested) by the animals are shown. Plant species which were tasted in amounts too low to be assigned to any weight, e.g. *Carya tomentosa*, and those which were refused are therefore not listed in Tab. B.5. The ultimate "classification" reflecting the sifaka's behaviour towards all species of trees, shrubs and climbing plants growing in the NHE-1 is given in Tab. B.7.

Tab. B.5: Daily intake and averaged amounts [g] ingested by group M&T in the NHE-1.

species	date (spring)						average[a]
	17.	18.	19.	20.	21.	22.	
Carpinus caroliniana	0.1	0.5			0.2		0.1
Robinia pseudoacacia				18.5		0.5	3.2
Diospyros virginiana		1.1					0.2
Quercus stellata	4.6		0.3	0.9	0.3		1.0
Acer rubrum	8.4	2.0	5.3	0.8	9.8	2.8	4.9
Morus rubra			2.5	18.4	25.2	2.3	8.1
Cercis canadensis		13.0		1.5	28.7	2.4	7.6
Quercus falcata	0.2	3.5	3.0		0.2		1.2
Liquidambar styraciflua	6.8	10.7	2.1	2.5	3.4	19.4	7.5
Liriodendron tulipifera			0.6		13.5	0.5	2.4
Vitis sp.		3.0			2.2	1.1	1.1
Fraxinus americana	2.0				0.5		0.4

[a] Average values include all days being in the forest, since food species were available during the entire observation time outside.

The amounts ingested in the forest could vary considerably from day to day (Tab. B.5), presumably because the group M&T was not habituated to the forest at the onset of this study. For details regarding the time spent eating in the forest and the estimated weights ingested by either focal animal of the group, see App. I.2.

Morus rubra, *Cercis canadensis*, and *Liquidambar styraciflua* were the most favoured tree species in the forested enclosure NHE-1. *Liquidambar styraciflua* and *Acer rubrum* were eaten every day when outside, whereas *Liriodendron tulipifera* and *Robinia pseudoacacia* were only occasionally eaten. Other plant species listed in Tab. B.5 were finally classified as being "tasted" (Tab. B.7).

According to the "classification system" (Tab. B.7) including all plant species growing in the NHE-1, 6 species were eaten, 7 were tasted and 4 spat out, and approximately 7 species were tasted daily by the group M&T.

Any change of the food begs the problem that animals might react with illness, especially diarrhoea. Tiberius had some loose stool on the 20th of May, which seemed to improve after three days. Reasons remain to be hypothesised. Faecal samples were analysed but no parasites or pathogenic bacteria could be found (staff, pers. comm.). The highly adapted gut system may have reacted to the changed environmental and feeding conditions combined with a different activity pattern during the day. Physical stress along with an accelerated gut passage rate might have also been reasons (Warner, 1981; Stevens, 1988).

Marcella seemed to loose some weight but did not react with soft stool. Initial loss of body weight of animals released into forested enclosures due to more activity combined with a better physical condition has already been described for other lemur species at the DULC. On the other hand, weight fluctuations also occur under natural conditions depending on seasonal food supply and animals are adapted to it (Charles-Dominique, 1979; Stevens, 1988; Glander *et al.*, 1992; Powzyk, 1997).

Group of Julian and Drusilla

In contrast to group M&T, group J&D had already been well habituated to the forest when the study was started (for a map see Fig. B.2, p. 22). Group J&D spent about 6.5 hours daily outside, which is nearly double the time per day of group M&T. Hence, group J&D must have already well known their territory with its species occurring, as it was not observed, that any species were spat out after being tasted. Therefore, in the autumn the study was focused on the species eaten. Compared to group M&T, only very few species were tasted by group J&D. Avoided and untouched plant species are not listed in Tab. B.6, but are included in Tab. B.7, and in App. II.2.

Tab. B.6: Daily intake and averaged amounts [g] ingested by group J&D in the NHE-3.

species	date (autumn) 04.	05.	06.	07.	08.	09.	10.	average[a]
Robinia pseudoacacia	2.4	2.0	1.0					0.8
Diospyros virginiana							6.5	0.9
Albizia julibrissin	0.8							0.1
Carya tomentosa	2.5	1.0				1.5		0.7
Pinus taeda, dry needles	1.0	0.2	0.2	1.0	1.0	2.0		0.8
Quercus sp.		0.6						0.1
Acer rubrum, leaves	9.6	9.6	12.8	26.4	16.0	35.2	2.4	16.0
Acer rubrum, stalks				0.9				0.1
Oxydendrum arboreum, leaves	0.3	1.5	2.5			2.5		1.0
Oxydendrum arboreum, bark	15.0	10.0						3.6
Liquidambar styraciflua	67.0	54.0	31.2	11.1	32.5	30.6	53.3	39.9
Liriodendron tulipifera	40.0	60.0	48.0	20.0	54.0	16.0	46.0	40.6
Vitis sp., leaves	2.0	20.0	38.0	5.6		8.0	3.2	11.0
Vitis sp., wood					24.0	21.0	18.0	9.0

[a] Average values include all days being in the forest, since food species were available during the entire observation time outside.

Most selected species were *Liriodendron tulipifera* and *Liquidambar styraciflua*, followed by *Vitis* sp. wood and leaves. A considerable amount of wood was eaten from *Vitis* sp. by both animals. *Acer rubrum* leaves and *Oxydendrum arboreum* bark and leaves were the next preferred food items within the forest. *O. arboreum* was the only tree species, of which more of the bark was eaten than of the leaves.

Quercus sp. leaves were only ingested once by one sifaka of group J&D. *Diospyros virginiana* and *Vitis* sp. were the only plant species that were treated in different ways by the study groups during the spring and the autumn season, albeit occurring within both natural habitat enclosures (Tab. B.7). The small amount eaten within the forest on the 7th of October, was attributed to the fact that animals were involved in the movie production and too excited and curious to find the calmness and rest to concentrate on feeding.

Robinia pseudoacacia was less eaten during the autumn than during the spring. Although *R. pseudoacacia* can only be categorised as being "tasted" by J&D, some leaves were eaten by Gordian. So, *R. pseudoacacia* is finally categorised as being eaten during the autumn.

3.2 Classification of plant species according to the feeding behaviour of *P. v. coquereli*

Events

Every single event of behaving toward a plant species was recorded. Evaluation of all single events concerning one plant species should result finally in the "classification system" (Tab. B.7) to correlate ultimately the feeding behaviour shown by the sifakas to the secondary plant compounds inherent to the respective plant species and plant parts (chapter C.II, App. II.2).

Single events were recorded as being tasted, spat out, eaten or passed with respect to individual plant species and their parts. A plant specimen is scored as being tasted, if only small amounts were ingested, normally about one bite, with a supposed weight of less than one gram. A food item was recorded as being eaten, if definitely more than one bite was ingested by either animal of the group. However, taking some plant part into the mouth does not implicitly mean to swallow it. Animals had to take the decision between tasting and swallowing a food item or spitting it out again.

Plants were scored as being passed, if animals were seen in or jumping through a tree but did not try to put any plant item into their mouth. In these cases, plants were just used as means for locomotion, e.g. sitting or climbing facilities.

Ultimately, scored events do not inevitably result into the same frame of "classification". All events had to be evaluated to result in a conclusive "classification system" (Tab. B.7).

Established classification

The evaluation of all single events results in the "classification system". The ultimate classification was performed separately for each season to reveal seasonal differences in treating plant species and plant parts. The following definitions are made to build a simple system to characterise all plant species of the forest including the browse species offered within the cages according to their obvious importance for the sifakas:

eaten	More than one bite was repeatedly ingested by either animal of a group, so that at least an estimated weight of 5 g was achieved.
tasted	An estimated weight of less than 5 g was finally ingested by one or both animals of a group.
refused	Plants were spat out by one or both animals of a group. Refusing a plant species was consistent for both animals of a respective group. A plant species was never more often tasted and swallowed than it was spat out.
passed	A tree species was passed, if it was used for locomotion activity such as climbing and sitting or just jumping through, and therefore being touched. It was never seen to be tasted as potential food source.
untouched	Plants were never seen to be touched although growing in the natural habitat enclosure of a group, sometimes even on the animal's track.

The main interest of the sifakas was granted to the leaves, so that this classification predominantly regards the leaves, but also other plant parts are included as far as they appeared during the observation seasons. It did not happen that one plant part, e.g. the bark, was refused after being tasted while another part, e.g. the leaves, were highly selected from the same species. The decision in favour of one or another part of the same species must have been taken on an earlier stage of recognition. Of some species, woody twigs were gnawed at but not to the exclusion of at least immature leaves. Moreover, it could happen, that a species was eaten in one season, but not in the other.

The decision how to treat the plant part tasted, e.g. to swallow the leaf or bark item or to refuse it after tasting was consistently taken by the focal lemurs during one season. On the other hand, treating individual parts of the same leaf species (leaf blade, leaf stalk) could differ individually.

The “classification system” (Tab. B.7) reflects the feeding pattern of the focal sifakas and thereby the importance of individual plant species and plant parts for the sifakas. Seasonal differences regarding the feeding pattern are indicated. Plants such as grass, herbs and bamboo, which were normally just traversed to reach a different site of the forested habitat enclosure are not included in this table. For the occurrence of these species in either natural habitat enclosure, see Fig. B.1, p. 21 and Fig. B.2, p. 22.
In Tab. B.7 plant species are listed alphabetically according to their plant family. Latin and vernacular names of each plant species and their respective family are presented. A short description of how the species were treated by the animals is included, and impressive events are recorded. Some notes characteristic of the species are added according to the “Audubon Society field guides to North American trees and wildflowers” (Little, 1993; Niering and Olmstaed, 1992). Notes on the plant’s odour and further characteristics, as well as on species not included in the field guides (*Ligustrum vulgare*, *Viburnum rafinesquianum* and *Vitis* sp.) are additionally given according to their appearance.

Tab. B.7: “Classification” of plant species and parts based on the feeding behaviour of the sifakas. Vernacular names and some important notes are included.

family, genus, species and descriptive notes	**classification**
Aceraceae: Maple Family	
Acer rubrum L.: Red Maple, Scarlet Maple, Swamp Maple Tips of stems with young leaves and reddish leaf buds were preferred, albeit animals ate medium sized and older leaves as well, and partly leafstalks and the leaf base remained at the twigs. Especially Marcella liked older leaves and green leafstalks. Particularly in the autumn it could be differed between eating leaf blades and leafstalks. One was eaten to the exclusion of the other. Height: 18-27 m. Large tree with red flowers, fruit, leafstalks, and autumn foliage. Opposite leaves; broadly ovate, with 3 shallow short-pointed lobes (sometimes with 2 smaller lobes near base); irregularly and wavy saw-toothed; long red or green leafstalks. Foliage turning red, orange, and yellow in autumn. Tough wood, no pith.	**leaves eaten**
Anacardiaceae: Cashew or Sumac Family	
Mangifera indica L., leaves: mango *Mangifera indica* L., fruits: mango Once in May, a branch of *Mangifera indica* was presented with an unripe fruit as browse to group M&T. Leaves of the branch were only tasted and mostly dropped. Both animals of group M&T preferred woody tips of twigs and stems and gnawed at it; and they liked the unripe fruit. Furthermore, animals received occasionally commercial mango fruits in the morning. Both, the unripe and the ripened fruits were liked.	**leaves tasted, fruit eaten**

family, genus, species and descriptive notes	classification
Rhus copallina L.: Shining Sumac, Dwarf Sumac, Winged Sumac Leaves of any age were eaten, e.g. leaf buds, young and old leaves, partly including the axis, throughout the year; only woody parts were left. Some bark and pith was noted to be ingested in autumn. One technique to eat the leaflets of *R. copallina* was similar to that of eating *A. julibrissin* leaves: stripping off the leaflets by means of their tooth comb starting from the stem-side. This was especially exhibited in spring, when leaflets easily came off the axis. Alternatively, the entire axis including the leaflets were eaten up, starting from the upper side. As third variant, especially used for autumn leaves, leaflets were bitten off singularly. Sometimes, animals changed their technique imperceptible while eating. In the autumn, aged *R. copallina* leaves were obviously too tough and rigid to strip leaflets easily off the axis. However, animals seemed to have the same problem harvesting the leaflets as I had. Height: 7.6 m. Shrub or small tree with a short trunk and open crown of stout, spreading branches. Leaves are pinnately compound; to 30 cm long; with flat broad-winged axis. 7-17 leaflets (27 in south-eastern variety), 2.5-8 cm long; lance-shaped; usually without teeth; slightly thickened; stalkless; shiny dark green above, paler beneath. Watery sap. Foliage turning dark reddish-purple in the autumn. Aromatic, resin-like excretions, especially on less lignified parts; pithy stems.	**leaves eaten**
Rhus radicans L.: Poison Ivy Height: vine. Upright climbing or trailing shrub; compound leaves; divided into three glossy or dull green long leaflets. That plant is extremely variable in form, occurring as a ground cover along roadsides, an erect shrub (especially in sandy coastal areas), or a large vine on trees. In the NHE-1 poison ivy occurred throughout as ground cover.	**untouched**
Betulaceae: Birch Family	
Carpinus caroliniana Walt: American Hornbeam, Blue-beech, Water-beech, Ironwood The leaves were tasted four times by Marcella and only once by Tiberius. Marcella bit into reddish young leaves; only small amounts were tasted. Height: 9 m. Elliptical simple leaves, sharply double saw-toothed, long-pointed at tip, turning orange to red in the autumn. The vernacular name ironwood covers both, the indigenous hop hornbeam, *Ostrya virginiana* (most commonly), and the hornbeam, *Carpinus caroliniana* (Crellin and Philpott, 1990).	**leaves tasted**
Caesalpiniaceae: Legume Family	
Cercis canadensis L.: Eastern Redbud, Judas-tree Upper leaves (young reddish leaf buds) were preferred. Leafstalks including the base of medium sized and older lower leaves were refused. In spring, pods were occurring on the tree which were also liked by the animals. Height: 12 m. Leaves are 6-11 cm long and broad; heart-shaped, with broad short point; without teeth; long-stalked; turning yellow in the autumn.	**leaves and pods eaten**
Caprifoliaceae: Honeysuckle Family	
Lonicera japonica Thunb.: Japanese Honeysuckle Japanese honeysuckle was often embracing other plants such as *R. copallina*. During the observation time in spring, Japanese honeysuckle was in flower. That vine was not eaten, neither in the cage, nor outside in the forest, not even the sweet tasting flowers in spring. Once Tiberius seemed to lick on the flowers. Height: vine. It has fragrant white tubular flowers, which are turning yellow with age. Flowering occurs from April to July, occasionally into the autumn. Sweet nectar can be milked from the base of the corolla. Opposite, ovate, untoothed, evergreen leaves.	**flowers tasted**
Viburnum prunifolium L.: Blackhaw, Stagbush, Sweethaw Shrub or small tree; height: 6 m. Opposite leaves; elliptical; finely saw-toothed. Turning shiny red in the autumn.	**untouched**

family, genus, species and descriptive notes	classification
Viburnum rafinesquianum: Rafinesque Viburnum Leaves were tasted twice by Marcella but she did not like them. No leaf was ingested. Shrub or small tree; leaves opposite, ovate; blunt or notched at base; sunken veins ending in large teeth; long leafstalks.	**leaves refused**
Cornaceae: Dogwood Family	
Cornus florida L.: Dogwood, Flowering Dogwood, Eastern Flowering Dogwood The leaves were only tasted by Tiberius and completely ignored by Marcella. Tiberius tried to eat them thrice, but twice they were spat out again. Height: 9 m. Opposite, elliptical simple leaves; edges slightly wavy, appearing not toothed but with tiny teeth visible under a lens; short-stalked; scarlet autumn foliage.	**leaves refused**
Cupressaceae: Cypress Family	
Juniperus virginiana L.: Red Juniper, Eastern Redcedar The tree was passed several times, but never seen to be tasted or eaten. Height: 12-18 m. Evergreen, aromatic tree; leaves opposite in four rows forming slender 4-angled twigs; leaves scalelike, not toothed; dark green, with gland-dot.	**passed**
Ebenaceae: Ebony Family	
Diospyros virginiana L.: Common Persimmon, Simmon, Possumwood The leaves were only seen to be ingested once by each animal of group M&T, but more than 5 g were eaten by group J&D in autumn. Height 6-21 m. Ovate to elliptical, long-pointed leaves; without teeth; slightly thickened; turning yellow in the autumn.	**leaves tasted in spring, eaten in autumn**
Ericaceae: Heath Family	
Oxydendrum arboreum (L.) DC.: Sourwood, Sorrel-tree, Lily-of-the-valley-tree Leaves and bark were ingested in the autumn. The bark, the leaves or only the leafstalks were ingested by the animals (event). Sometimes, animals seemed to be astonished because of the strange taste of the leaves. The bark was only scraped off the younger twigs. *O. arboreum* did only occur in the NHE-3. Height: 15 m. Elliptical or lance-shaped, finely saw-toothed long leaves. Despite the sour taste of the foliage, sourwood honey is esteemed. Foliage turning red in the autumn.	**leaves tasted, bark eaten**
Fabaceae: Legume Family	
Robinia pseudoacacia L.: Locust, Yellow Locust, Black Locust Upper younger leaves were preferred in contrast to leaves at lower parts of the twigs. This might be due to strong spines growing at nodes, which were only smooth in the upper part of the twig. Height: 12-24 m. Medium-sized, spiny tree. Pinnately compound leaves with 7-19 leaflets; paired (except at end); elliptical; with tiny bristle tip; without teeth; drooping and folding at night. Twigs with stout paired spines at nodes; very fragrant flowers.	**leaves eaten**
Fagaceae: Beech Family	
Quercus falcata Michx.: Southern Red Oak, Spanish Oak, Swamp Red Oak Similar to *Q. stellata*, the bark was tasted and occasionally eaten (event). Young leaves were preferred by both animals of group M&T, occasionally medium sized leaves were ingested (event). Of older leaves, only the tips were eaten, the base was left attached to the tree. In the autumn, preferentially leafstalks were ingested, while the leaf blades were dropped. The leafstalks tasted slightly astringent to me, were moist and of aromatic odour. The sifakas preferred the lower thickened parts of the leafstalks which were close to the stem. Height: 15-24 m. Elliptical leaves, deeply divided into long narrow end lobe and 1-3 shorter mostly curved lobes on each side, with 1-3 bristle-tipped teeth; sometimes slightly triangular	**bark and leaves tasted**

family, genus, species and descriptive notes	classification
with bell-shaped base and three broad lobes; turning brown in the autumn.	
Quercus phellos L.: Willow Oak, Pin Oak, Peach Oak Height: 15-24 m. Narrow oblong or lance-shaped leaves with tiny bristle-tip; without teeth; edges straight or slightly wavy; turning pale yellow in the autumn.	**untouched**
Quercus stellata Wangenh.: Post Oak, Iron Oak The bark was tasted once by Tiberius, leaves were ingested in small amounts by both animals of group M&T. Mainly young leaves and leaf buds were ingested. *Q. stellata* was only tasted once in the autumn. Height: 9-21 m. Obovate simple leaves with 5-7 deep broad rounded lobes, 2 middle lobes largest, with short-pointed base and rounded tip, slightly thickened, turning brown in autumn.	**bark and leaves tasted**
Hamamelidaceae: Witch-Hazel Family	
Liquidambar styraciflua L.: Sweetgum, Redgum, Sapgum Only some leaf buds and upper younger leaves were eaten when *L. styraciflua* was offered within the cage, the base and leafstalk of older leaves remained attached to the twigs. Occasionally, leaves were spat out again when eaten inadvertently. Green stems (2-9 cm) were also eaten from the tips downwards. In the forest, the bark was tasted once by Marcella; the small and medium sized leaves were eaten, young leaves were preferred. Once Tiberius dropped some leaves instead of eating them; Marcella dropped the tips of older leaves; only in the forest she ate the base of older leaves. In the autumn, both, coloured and dry leaves were eaten. Finally, eating *L. styraciflua* leaves varied considerably, albeit the individual preferences described could only be observed outside in the forest, not within the cage. Height: 18-30 m. 7.5-15 cm long and wide, star-shaped or maple-like leaves with 5, sometimes 7, long-pointed, finely-saw-toothed lobes; notched base. Resinous odour when crushed. Slender leafstalks, nearly as long as blades. Leaves are turning reddish in the autumn. Exhibits a strong aromatic odour, especially when leaves were stripped off; in autumn quite lignified, no pith.	**leaves eaten**
Juglandaceae: Walnut Family	
Carya tomentosa (Poir.) Nutt.: Mockernut Hickory, White Hickory, Mockernut In spring, only one leafstalk was ingested by Tiberius. In autumn, leaves were tasted. Height: 15-24 m. Pinnately compound, long leaves, 7 or 9 leaflets, elliptical or lance-shaped, finely saw-toothed; nearly stalkless. Turning yellow in the autumn.	**leaves tasted**
Magnoliaceae: Magnolia Family	
Liriodendron tulipifera L.: Tuliptree, Yellow-poplar, Tulip-poplar In both seasons leaves were eaten. No individual preferences could be observed. Some pith was noted to be eaten additionally in the autumn. Height: 24-37 m. One of the tallest and most beautiful eastern hardwoods. Leaves are 7.5-15 cm long and wide; blades of unusual shape, with broad tip and base nearly straight like a square, and with 4 or sometimes 6 short-pointed paired lobes; long-stalked; turning yellow in the autumn. Blades have a broad tip, the base is nearly straight like a square; four, sometimes six short-pointed paired lobes. Aromatic, spicy odour when leaves were stripped off; pithy stems.	**leaves eaten**
Mimosaceae: Legume Family	
Albiz(z)ia julibrissin Durazzini: Mimosa-Tree, Silktree, Powderpuff-tree In May all leaves were eaten, but the younger leaves were preferred. In the autumn only the younger leaves were selected. The sifakas stripped the leaflets off the axis by means of their tooth comb starting from the stem-side (lower side of the axis), while fixing the twig by one of their hands. Axis were mostly refused and dropped when they got inadvertently into their mouths. In May, tips of stems with leaf buds were not always eaten, whereas in the autumn the smooth tips were ingested. In the autumn, leaflets were very firm and depending on age not easy to be stripped off. Obviously, animals had the same difficulties and leaflets were torn off the axis.	**leaves eaten** (heavily eaten in spring, less eaten in the autumn)

family, genus, species and descriptive notes	classification
Height: 6 m. Native from Iran to China. Small ornamental with short trunk or several trunks and a very broad, flattened crown of spreading branches and with showy pink flower clusters. Leaves are bipinnately compound; 15-38 cm long; fernlike; with 5-12 pairs of side axes; 15-30 pairs of oblong pale green leaflets are attached to each axis which fold up at night. Flowering throughout summer. Strong odour of the firm pith.	
Moraceae: Mulberry Family	
Morus rubra L.: Red Mulberry, Moral The tree only occurred within the NHE-1. The leaves were eaten in different ways. Marcella preferred to eat the tips of the leaves or the entire blade, but avoided the leafstalks and "latex". She was eating both, young and older leaves. On the contrary, Tiberius seldom ate the entire leaf, he typically bit once into the leafstalk including the base, licked off the milk coming out of the stalks and dropped that leaf. For instance, on one day he bit exactly 38 times into leafstalks and bases, licked off the "latex" and dropped all 38 leaves. Once both were gnawing at the wood of twigs and branches. However, young leaves were preferred. Height: 18 m. Leaves are in 2 rows, ovate, abruptly long-pointed, with 3 main veins from often unequal base, coarsely saw-toothed. Often with two or three lobes on young twigs. Turning yellow in the autumn.	**leaves eaten**
Nyssaceae (according to Hegnauer (2001)) or **Cornaceae: Dogwood Family (according to Little (1993))**	
Nyssa sylvatica Marsh.: Black Tupelo, Blackgum, Pepperidge Height: 15-30 m. Elliptical or oblong leaves; not toothed (rarely with a few teeth); slightly thickened; turning bright red in early autumn.	**untouched**
Oleaceae: Olive Family	
Fraxinus americana L.: White Ash One tree of that species grew next to the holding cage within the NHE-1, so that the lemurs passed nearly every day that tree for several times on their route through the forest. Nevertheless, it was only eaten once and tasted twice (events). Height: 24 m. Opposite, pinnately compound ovate or elliptical leaves, usually 7 (5-9) leaflets paired, except at end, finely saw-toothed or almost without teeth, turning purple or yellow in the autumn.	**leaves tasted**
Fraxinus pennsylvanica Marsh.: Green Ash, Swamp Ash, Water Ash Height: 18 m. Opposite, pinnately compound leaves; 5-9 (usually 7) leaflets; paired (except at end); lance-shaped or ovate; coarsely saw-toothed or almost without teeth; turning yellow in the autumn.	**untouched**
Ligustrum vulgare: Common Privet, European Privet The leaves were tasted once by either animal of group M&T on different days, but were spat out immediately by both animals. Shrub or small tree; opposite, elliptical or ovate simple leaves; short leafstalks.	**leaves refused**
Pinaceae: Pine Family	
Pinus taeda L.: Loblolly Pine Only dead brown needles were seen to be ingested, three at once, since the needles grow in bundles. Needles were eaten either when fallen into the holding cage, or while the sifakas were sitting in the tree. Height 24-30 m. Evergreen conifer, among the fastest-growing southern pines. Long needles; three in bundle; stout, stiff, often twisted; green; fragrant resinous foliage.	**tasted (dry needles)**
Rosaceae: Rose Family	
Photinia glabra Maxim.: Photinia, Red Tip Only the upper reddish leaves (according to the vernacular name "red tip") were preferred.	**leaves eaten**
Prunus serotina Ehrh.: Black Cherry, Wild Cherry, Rum Cherry	**untouched**

family, genus, species and descriptive notes	**classifi-cation**
Height: 24 m. Crushed foliage and bark have distinctive cherry-like odour and bitter taste. Elliptical leaves with 1-2 dark red glands at base; finely saw-toothed with curved and blunt teeth; slightly thickened. Turning yellow or reddish in the autumn.	
Simaroubaceae: Quassia Family	
Ailanthus altissima (Mill.) Swingle: Tree-of-Heaven This tree was passed three times during 6 days by group M&T. Although it was passed by both animals, only Marcella seemed to taste some plant tissue. (It could not be exactly observed.) Height: 15-24 m. Male flowers and crushed foliage have disagreeable odour. Leaves are pinnately compound; 13-25 leaflets (sometimes more), paired (except at end); broadly lance-shaped, with 2-5 teeth near broad 1-sided base and gland-dot beneath each tooth.	**passed**
Ulmaceae: Elm Family	
Ulmus alata Michx.: Winged Elm, Cork Elm, Wahoo Leaves were only tasted by Marcella, but she did not like it. Once, she spat it out. Height: 12-24 m. Elliptical leaves, often slightly curved with sides unequal, doubly saw-toothed, thick and firm; in two rows; turning yellow in the autumn. The name refers to the distinctive broad corky wings present on some twigs.	**leaves refused**
Vitaceae	
Vitis cinerea Engelm.: } *Vitis* sp.: grape *Vitis rotundifolia* Michx.: } Leaves were eaten of both species. As species could not always be exactly distinguished when eaten in high trees with dense foliage, the amounts eaten of both species were assigned to *Vitis* sp. Woody twigs were eaten once by Marcella, presumably *V. cinerea*. In the autumn, wood and leaves were heavily eaten by both animals of group J&D. *Vitis cinerea*: Height: vine. Heart-shaped, saw-toothed simple leaves. *Vitis rotundifolia*: Height: vine. Rounded, coarsely toothed simple leaves.	**in spring: wood and leaves tasted** **in the autumn: wood and leaves eaten**

According to Tab. B.7, no special appearance (e.g. leaf texture, toughness, size) of the plant tissue selected or refused can be figured out as possible criterion for food selection. The taste of the preferred leaf species, that were fed in spring as browse, is described in App. I.3. Hence, a literature review on the secondary plant compounds of these plant species dealt with (chapter C.II and App. II.2) will be the basis for subsequent chemical and pharmacological analyses (see chapters C.III-VI).

3.3 Quantitative evaluation of the food eaten

After characterisation of the plant species and plant parts reflected by the feeding behaviour, now food intake will be quantified. The quantities ingested will be monitored on a fresh weight basis. During the observation time in the forest every amount eaten was recorded. Hence, the weight eaten in the cages is completed by the weight of plants eaten in the forest.

All weights ingested are referred to the entire group comprising one or two focal lemurs as well as to bodyweight[1]. Hence, different bodyweights of individual sifakas and their different ages are taken into account.

[1] The amount (g) of any food source eaten by each group is divided by the sum of bodyweights (kg) of all members of the respective group.

Quantification of the food composition will be the basis for a subsequent consideration of nutrient and energy supply of individual food resources (chapter C.I), as well as for pharmacological aspects of the food due to chemical plant compounds inherent to the leaves (chapters C.III-VI).

Food composition of the focal lemur groups

The food of the sifakas was composed of three major food categories: a portion of leaves, a large variety of fruits and vegetables and commercial leaf-eater primate chow. In the following consideration, emphasis is put on the overall feeding pattern taking into account different holding conditions, different ages of the study animals as well as seasonal constraints. Particular respect is dedicated to the leaf species selected by the animals with respect to species composition, elucidation of preferentially eaten species and seasonal fluctuations. Although to a minor extent some other plant parts (wood and bark) were ingested as well (Tab. B.7), all plant tissue apart from fruits and vegetables is noted as "leaves" due to their predominance (Fig. B.3).

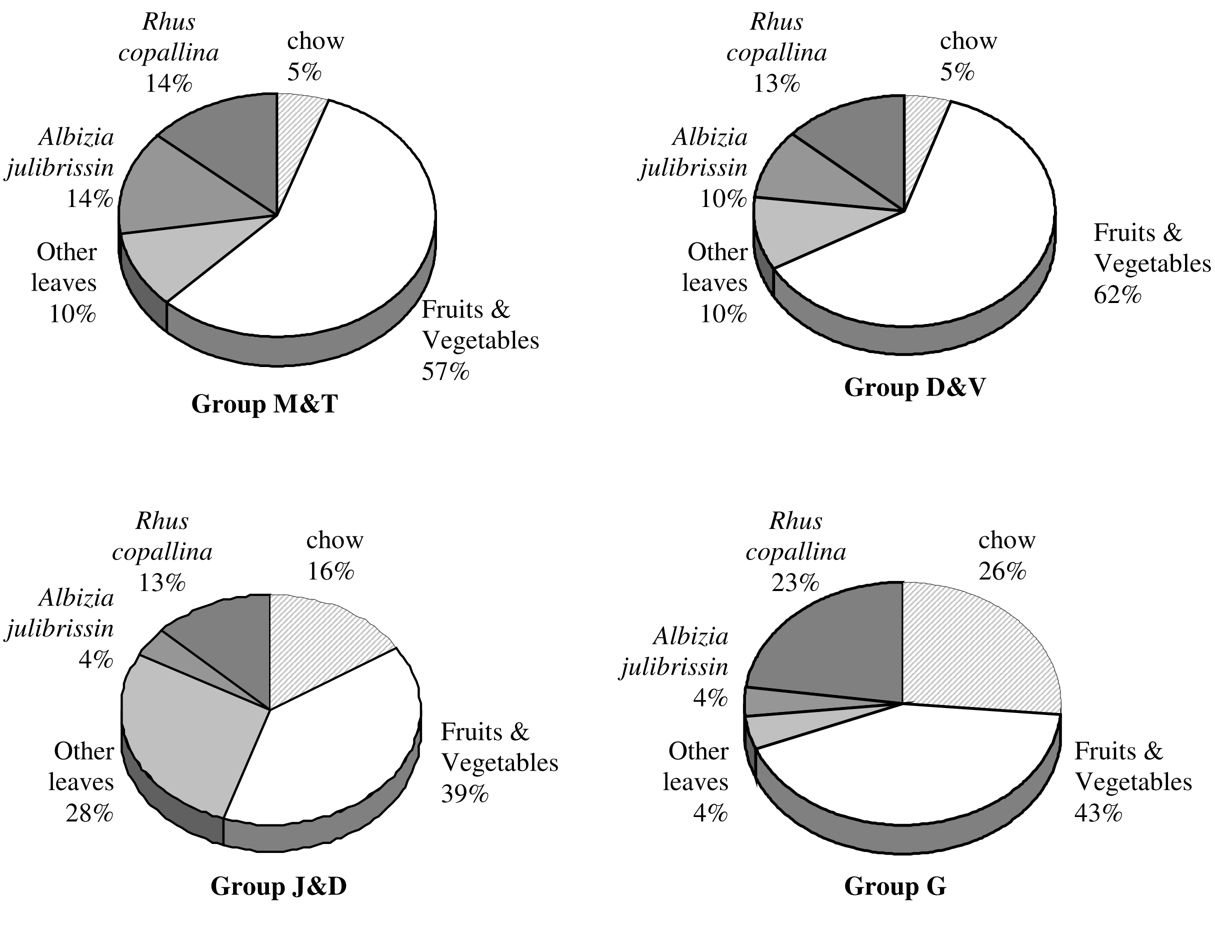


Fig. B.3: Food composition in weight % of the four study groups on a fresh weight basis, and averaged on the entire study time. "Other leaves" (Tab. B.8) designate all leaf species except for the leaves of *Rhus copallina* and *Albizia julibrissin*, including the weights of plant species eaten in the forest.

In Fig. B.3, the weight percentages of the three major food resources are reviewed respectively for the four study groups. Weights of the plant species ingested in the forest are included in this calculation. "Other leaves" give the percentages of all leaf species ingested apart from *R. copallina* and *A. julibrissin* leaves. Leaf species which were ingested for at least 1 % of weight (and more) are explicitly listed in Tab. B.8, whereas amounts of less than 1 % of weight are summarised as "rest".

Tab. B.8: "Other leaf species" of Fig. B.3 account for the percentages of all other leaf species ingested by each lemur group apart from the leaves of *R. copallina* and *A. julibrissin*. Leaf species that were ingested for at least 1 % (or more) are explicitly listed, whereas amounts of less than 1 % are included in the "rest".

other leaf species	**M&T**	**D&V**	**J&D**	**G**
Liquidambar styraciflua	2%	3%	9%	1%
Cercis canadensis	2%	4%		
Acer rubrum	5%	3%	4%	
Vitis sp.			2%	
Liriodendron tulipifera			9%	2%
Photinia glabra				1%
rest	1%		4%	

It can be stated that the major portion of the food eaten consisted of fruits, vegetables and primate chow (Fig. B.3). However, the percentages of the leaves, fruits and vegetables and chow varied considerably between the four study groups.

Considering the entire portion of leaves, it is striking that those groups with access to natural habitat enclosures consumed a larger portion and a greater variety of leaves than those groups exclusively relying on the browse provided within the cages. While an average of 38 % leaves were consumed by group M&T and 45 % by group J&D, on average 33 % and 31 % were consumed by group D&V and Gordian, respectively. Overall, at least one third of the total fresh food was allotted to the leaves, with no apparent dependence on the animal's age.

Nearly equal percentages were eaten of *R. copallina* and *A. julibrissin* leaves by the groups in spring, while a dramatic decrease for *A. julibrissin* leaves can be stated for the autumn groups. In contrast, *R. copallina* was highly selected during both, the spring and the autumn season, which resulted in an overwhelming percentage within Gordian's diet.

The larger portion of leaves ingested by the lemurs with outdoor access may be due to the fact that the forest builds a more natural environment and animals had the possibility to a more selective choice as to leaf quality and quantity, than had caged animals. Especially in the autumn younger leaves were more abundant in the forest than could be provided by a bunch of browse within the cages, and animals apparently preferred the younger leaves to older ones of various species. Furthermore, it has to be kept in mind that in contrast to group M&T, group J&D was already well habituated to the forested habitat enclosure at the onset of this study and that they stayed outside for a longer period of time each day than group M&T.

Fig. B.4 reflects the food selection as to the leaf species presented as browse within the cages during the spring season. *R. copallina* and *A. julibrissin* leaves were clearly preferred.

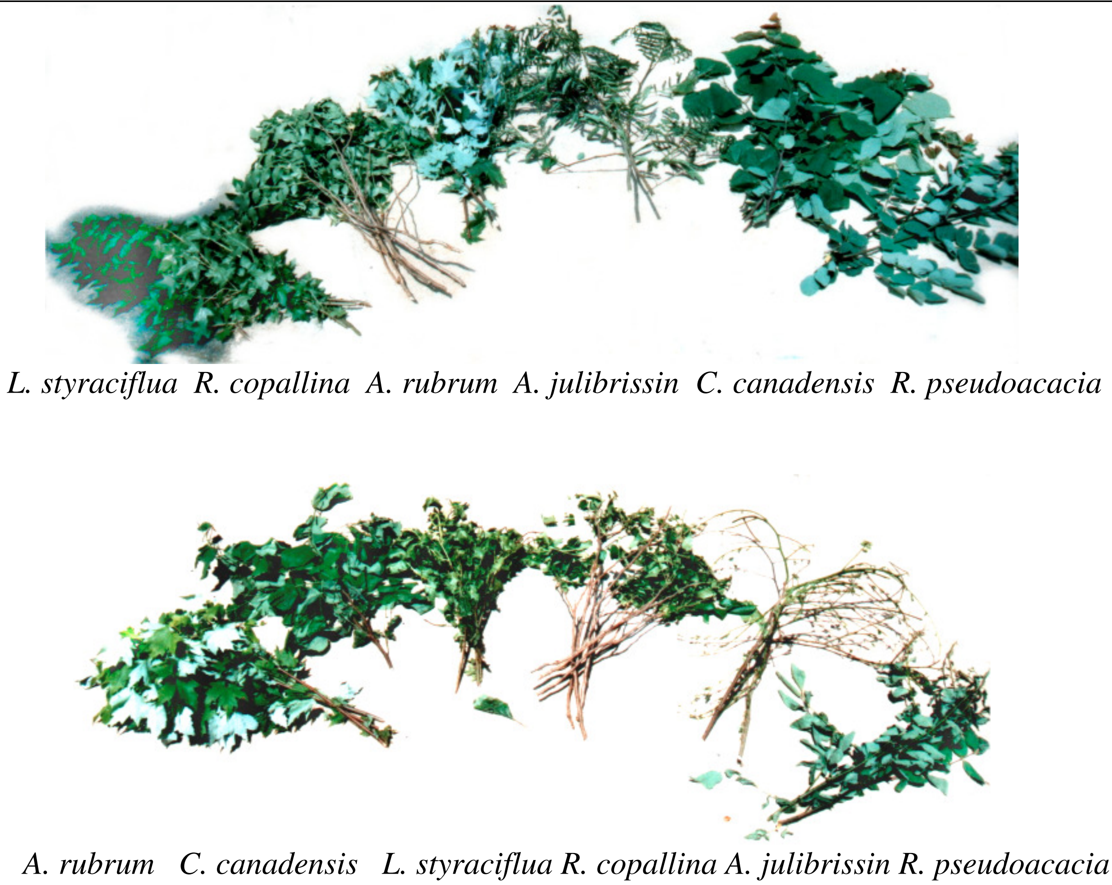


Fig. B.4: Feeding pattern of the focal lemur groups during the spring season: leaves before (upper photo) and after (lower photo) selection of the browse within the cages.

However, relations of weight % do not reflect the actual amounts eaten per group and per bodyweight. Since the weighing method is based on food intake of the entire group, the averaged quantity ingested is also referred to kg bodyweight (Fig. B.5 and Tab. B.9) to make food intake of individual animals with different bodyweights comparable to one another.

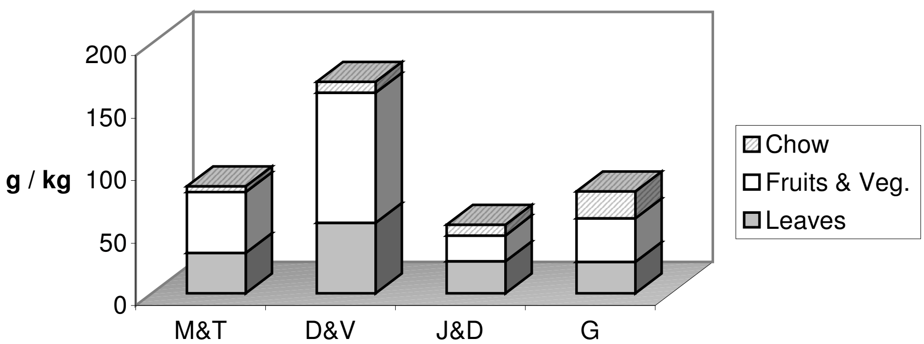


Fig. B.5: Weight eaten per kg bodyweight and day, respectively for each lemur group.

Thus, according to Fig. B.5 and Tab. B.9 the largest amount of leaves was ingested by group D&V referred to the entire group as well as to bodyweight. Comparing groups with adult and young animals separately to each other, an overall decrease in food intake can be stated for the groups in the autumn compared to the groups in spring. Referred to bodyweight (Fig. B.5 and Tab. B.9), in the autumn nearly half the total amount was eaten compared to spring, if groups with animals of comparable age are considered, respectively. If the total weight eaten per bodyweight is compared between adult and juvenile / subadult groups of either season, it is striking that nearly double the amount is eaten by groups with growing animals compared to adults. Group D&V consumed the largest amount of fruits & vegetables and leaves of all study groups. Group J&D being outside in the forest for most hours of each day consumed the smallest portion of fruits and vegetables per bodyweight of all four groups, and less leaves (by weight) than group M&T, albeit they had the largest percentage of leaves (Fig. B.3).

Tab. B.9: Food intake is given for each designated food source as total intake (g) referred to each lemur group (left column), and referred to bodyweight (right column). Estimated weights of food species eaten in the forest are included[a].

	total intake in g				**intake in g per kg bodyweight**			
	spring		**autumn**		**spring**		**autumn**	
food source	**M&T**	**D&V**	**J&D**	**G**	**M&T**	**D&V**	**J&D**	**G**
R. copallina[b]	100.6	134.8	59.8	55.7	11.8	22.7	7.0	18.6
A. julibrissin[b]	100.3	98.7	19.8	11.1	11.8	16.6	2.3	3.7
A. rubrum[b]	39.4	35.0	16.0		4.6	5.9	1.9	
C. canadensis[b]	18.3	54.8	0.0	2.3	2.1	9.2	0.0	0.8
L. styraciflua[b]	11.9	34.8	39.9	4.7	1.0	2.9	0.1	0.5
R. pseudoacacia[b]	8.4	17.5	0.8	1.5	1.4	5.9	4.7	1.6
L. tulipifera[b]	3.7		40.6	19.7	0.4		4.8	6.6
P. glabra[b]			24.5	28.9			2.9	9.6
total leaves[c]	276.2	337.6	218.6	76.6	32.4	56.7	25.6	25.5
fruits & vegetables[d]	416.9	620.7	179.1	104.0	48.9	104.3	21.0	34.7
chow[e]	39.1	51.5	73.8	65.0	4.6	8.7	8.7	21.7
total[f]	732.3	1009.7	471.5	245.6	85.8	169.7	55.3	81.9

[a] This table will be used to calculate dosages of identified plant compounds, that were ingested by the sifakas (see chapter C.VI).

[b] Weights of leaf species eaten, averaged only on the number of days when the respective food source was provided.

[c] Total amount of leaves ingested including all leaves irrespective of species, averaged on all study days. “Total leaves” are differently calculated from individual leaf species listed above and the value is not equal to the sum of those species.

[d] Total weight of fruits and vegetables eaten, averaged on all study days.

[e] Total weight of chow eaten, averaged on all study days.

[f] Total amount of fruits & vegetables, chow and leaves eaten, averaged on all study days.

Applying statistical methods to food resources provided and eaten and to compare the different study groups of different ages and different seasons to one another, following correlations can be figured out:

Fruits & vegetables including the portion of chow were statistically differently eaten on a high level of likelihood if all groups are compared to one another ($p < 0.01$, U-test, two-sided). One major reason for the difference in eating varying portions of fruits & vegetables and chow is apparently to search in its availability. While the groups in spring ate an average of 91 % (group M&T) and 99 % (group D&V) respectively of the chow provided, groups in the autumn could virtually eat chow *ad libitum* because nearly four times as much were provided. The opposite was valid for the supply of fruits and vegetables. While the groups in spring could eat fruits and vegetables nearly *ad libitum*, only one third of this quantity was provided for the groups in the autumn, which resulted in an intake of about 80 % by group J&D and 95 % by Gordian. Hence, groups in the autumn obviously compensated the large portion of chow for the low intake of fruits and vegetables due to the availability of the respective food resources. The large portions of chow ingested in the autumn resulted in a lower weight of overall food intake because of the high nutrient density of the chow. The different weights eaten between groups of spring and autumn and the different food composition chosen as regards portions of chow, fruits and vegetables can be explained if their nutrient and energy contents are taken into account (see chapter C.I).

Comparing the total weights of fruits and vegetables including the primate chow ingested to the respective amounts brought into the cage, 54 % and 64 % were eaten respectively by group J&D and group M&T, while about 80 % were eaten by the groups D&V and G. Applying the Spearman-rank-correlation test to fruits & vegetables including chow offered related to the daily amount eaten, significant correlations are found for the groups D&V and G ($r_s = 0.928$, $p < 0.01$, $n = 7$; $r_s = 0.745$, $p < 0.01$, $n = 10$, respectively), but not for the groups M&T and J&D. This corroborates the importance of fruits, vegetables and chow as food source for growing animals, as they ate nearly all food provided.

Taking into account observations during the feeding bouts, following results are obtained: All groups ingested nearly half the quantity of fruits & vegetables and chow during the observed feeding bouts referred to the corresponding amounts eaten during the day.

In consideration of the huge variability in number and quantity of fruits & vegetables offered to the groups, it is not possible to figure out any general or individual preferences. Of course, juicy fruits were preferred and typically eaten at first which might have to do with their water content. In contrast to the autumn observations, in spring animals were never seen drinking water.

In contrast to the various species of fruits and vegetables, clear general, seasonal and individual preferences can be figured out for the different leaf species. In the following, the pattern of leaf selection shall be further investigated. Especially dependencies on supply and demand, but also on the animal's age and seasons shall be considered.

Correlations between the amount of individual leaf species brought into the cage and the quantities eaten by the groups are depicted as diagrams in Fig. B.6 to Fig. B.11. As far as the Spearman-rank-correlation test yielded statistically significant dependencies, values are additionally listed. Applying the Spearman-rank-correlation test, it should be found out, which of the plant species provided as browse within the cages were highly preferred and which were just occasionally eaten or selected only in small amounts. In cases of indifference, a correlation would be expected irrespective of the plant species. (The weights eaten in the forest by the groups with outdoor access could not be included in this test, because no values do exist for the quantity of the food available.) The following results reveal a predilection for only few plant species in general, clear seasonal differences, and corroborate indirectly individual food preferences.

Fig. B.6 shows a strong Spearman-rank correlation between the weight offered and ingested of *R. copallina* leaves for the four lemur groups. Strongest correlations are found for groups with juvenile and subadult animals. However, based on bodyweight (Tab. B.9), the absolute amount eaten of *R. copallina* leaves decreased slightly for the autumn groups compared to the groups in spring, which might be interpreted as following the overall trend of eating less leaves during the autumn. Group J&D ate the least amount of *R. copallina* leaves, albeit sufficient *R. copallina* leaves had been provided. Group J&D only ingested about 50 % of the *R. copallina* leaves offered, while all other groups ingested averages between 74 % and 88 %, respectively. Although the reason remains somewhat obscure as to why group J&D ate the smallest amount of *R. copallina* leaves per bodyweight, the percentage (m/m) of *R. copallina* leaves eaten by J&D is comparable to percentages eaten by the groups in spring (Fig. B.3). The largest portion of *R. copallina* leaves was consumed by Gordian in the autumn, but this predominance of *R. copallina* in his diet may account for the limited choice of leaf species offered to him.

group	r_s	prob. of error	n
M&T	0.692	$p < 0.01$	12
D&V	1	$p < 0.001$	7
J&D	0.809	$p < 0.02$	8
G	0.918	$p < 0.001$	11

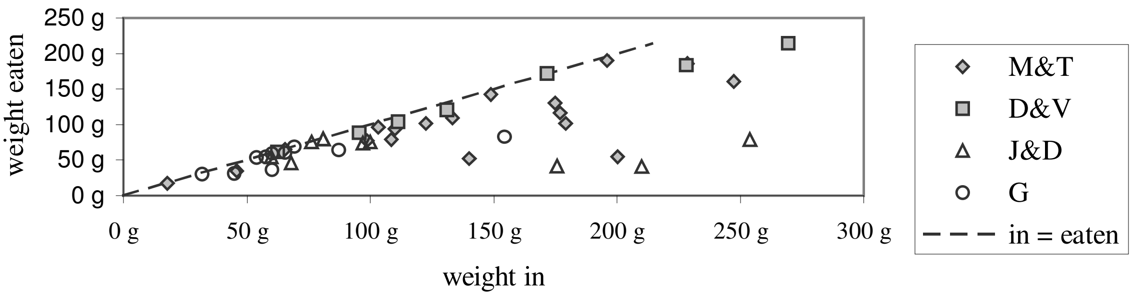


Fig. B.6: Correlation table and diagram between the weight of *Rhus copallina* leaves put into the cage and the amount ingested by each study group.

According to the Mann-Whitney U-test applied to weights eaten per bodyweight, differences in eating *R. copallina* cannot be found for groups of the same season, but for groups of different seasons (M&T related to J&D: $p < 0.02$, U-test, two-sided; D&V related to Gordian: $p < 0.01$, U-test, two-sided). Overall, *R. copallina* remained the most preferred leaf species during both seasons by all study groups.

Correlations for *A. julibrissin* leaves are depicted in Fig. B.7. For *A. julibrissin* leaves, a strong correlation could only be figured out for the leaves eaten in spring.

Whereas in spring 70-80 % of the *A. julibrissin* leaves offered were eaten, only less than 20 % were eaten in the autumn. Nevertheless, *A. julibrissin* was quite often ranking among the first ten species in both, the spring and the autumn season (App. I.1).

The most striking difference is found for the pattern in eating *A. julibrissin* leaves, if the quantities eaten per bodyweight are compared between the groups of different seasons. While statistically significant differences in eating *A. julibrissin* leaves are found if each group in spring is paired with each group in the autumn, respectively ($p < 0.01$, U-test, two-sided), no significant difference is found, if groups of the same season are compared to one another. If the amount eaten of *A. julibrissin* leaves is based on bodyweight, only about one fifth was eaten in the autumn compared to spring. It is reasonable to assume that the remarkable decrease in eating *A. julibrissin* leaves in autumn is due to the occurrence of more old leaves with a changed leaf composition, which was not tolerated by the animals. (Changes in the leaf composition as to primary and secondary plant compounds will be investigated in chapter C.I and C.IV, respectively.)
While in spring young leaves were abundant, only a small portion of *A. julibrissin* leaves

during the autumn consisted of younger leaves. Observations of the feeding behaviour support the strong predilection for the younger leaves during the autumn. It remains to be hypothesised how much *A. julibrissin* leaves animals would have eaten, if enough of the desired younger leaves had been provided within the cages.

group	r_s	prob. of error	n
M&T	0.846	$p < 0.001$	12
D&V	1	$p < 0.001$	7

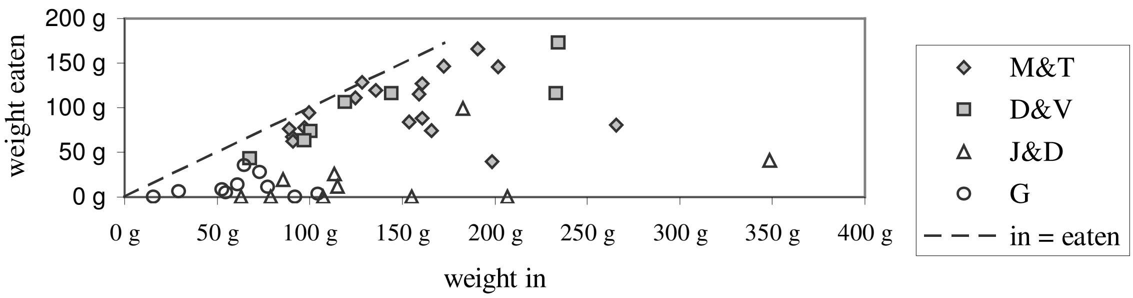


Fig. B.7: Correlation table and diagram between the weight of *Albizia julibrissin* leaves put into the cage and the amount ingested by each study group.

Comparing the quantities eaten between *R. copallina* and *A. julibrissin* within the four lemur groups, only within groups J&D and G significant differences can be found ($p < 0.01$, U-test, two-sided, respectively). That means, whereas *R. copallina* and *A. julibrissin* were not differently eaten from each other in spring by group M&T and group D&V, a statistical significant difference can be figured out for the feeding behaviour of the groups in the autumn.

In spring, both *R. copallina* and *A. julibrissin* leaves were generally more eaten than any other plant species. Especially in spring, on some days the same amount was eaten as offered in the cage and most part of *R. copallina* and *A. julibrissin* leaves provided were already eaten during the main feeding bouts. In both seasons, *R. copallina* and *A. julibrissin* leaves were most often ranking within the first ten different species eaten during the main feeding bouts (App. I.1). Except for individually preferred leaf species, normally less than half the amount of the other leaf species was eaten during the feeding bouts, if the amounts observed as being eaten are compared to the amounts eaten that day. During the feeding bouts in the autumn, Gordian's leaf eating consisted typically of *R. copallina* and *A. julibrissin* leaves. Observational data about struggles for *R. copallina* and *A. julibrissin* leaves and searching for any remaining leaf fragment on otherwise stripped branches corroborate the importance of both species within the sifakas' diet. The weights eaten of these favourites might have been higher, if more leaves to their liking had been provided.

Some of the other plant species were individually differently eaten. If the ranks of the first ten species are examined for each sifaka (Tab. I.1, p. 206), a predilection for *A. rubrum* leaves by Marcella (Fig. B.8) as well as for *C. canadensis* leaves by Valentinian (Fig. B.9) can be stated. Valentinian and Marcella accounted most for the individual leaf preferences revealed.

For group M&T a strong correlation between *A. rubrum* leaves provided in the cage and the amount eaten can be found (Fig. B.8). Especially Marcella liked to eat the reddish leaf buds and young leaves of *A. rubrum* tree. Within group M&T, *A. rubrum* was the next big portion of leaves eaten (5 %; Tab. B.8), when averaged on the study time. Outside in the forest, *A. rubrum* leaves were eaten every day, so that the reduction in eating *A. rubrum* during the last week within the cage was compensated by the amount selected in the forest. Within the cage, approximately 40 % of *A. rubrum* leaves offered were eaten by group M&T.

group	r_s	prob. of error	n
M&T	0.754	$p < 0.005$	11

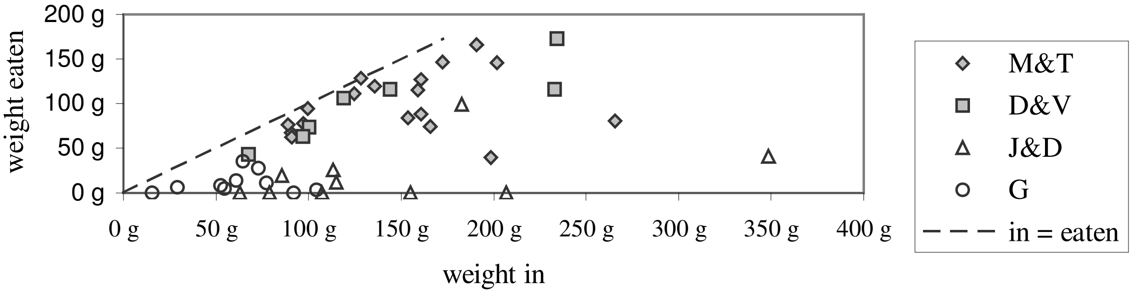


Fig. B.8: Correlation table and diagram between the weight of *Acer rubrum* leaves put into the cage and the amount ingested by each study group.

The predilection for *A. rubrum* leaves by the group M&T is corroborated statistically in many respects. Within group M&T, *A. rubrum* leaves were significantly differently eaten from most other tree species (from *R. copallina*, *A. julibrissin* and *L. tulipifera* $p < 0.01$, U-test, two-sided; from *R. pseudoacacia* $p < 0.05$, U-test, two-sided).

In contrast to spring, during the autumn session *A. rubrum* was not provided as browse within the cages and only available in the forest. Therefore, a conclusive evaluation for *A. rubrum* leaves remains sophisticated. In spring, it was much eaten, equally within the cages and outside in the forest, and as mentioned, it was a preferred food plant of Marcella. Referred to bodyweight, group J&D ingested nearly half the amount eaten by group M&T (Tab. B.9), although both groups show the same group-bodyweight. On the contrary, if only the feeding behaviour of the groups M&T and J&D exhibited in the forest is compared, more was eaten during the autumn than during the spring. In group J&D, *A. rubrum* contributed on average 4 % to the entire food eaten.

group	r_s	prob. of error	n
M&T	0.527	$p < 0.1$	10
D&V	0.9	$p < 0.01$	5

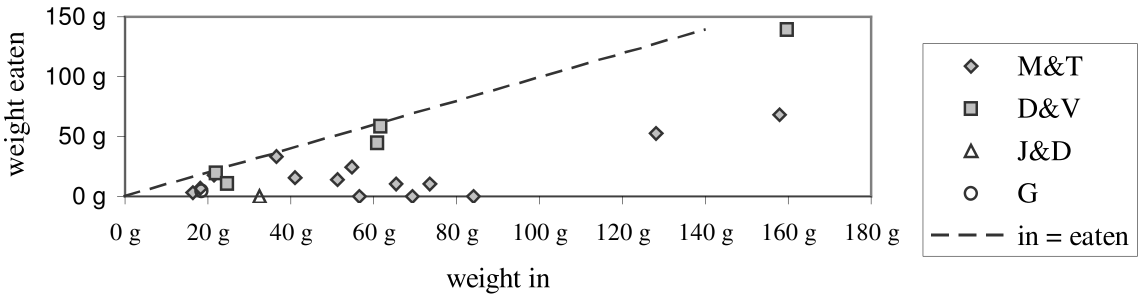


Fig. B.9: Correlation table and diagram between the weight of *Cercis canadensis* leaves put into the cage and the amount ingested by each study group.

Approximately 80 % of *C. canadensis* leaves offered were eaten by group D&V. *C. canadensis* amounted to 4 % within group D&V's diet and was statistically differently eaten only from *R. copallina* leaves ($p<0.05$, U-test, two-sided). Within group M&T, on average 2 % *C. canadensis* leaves were eaten (Tab. B.8), and outside in the forest it was one of the most selected leaf species (Tab. B.5). A significant Spearman-rank correlation is figured out for both groups in spring.

While *C. canadensis* was regularly fed to the groups in spring, in the autumn only twice it was brought into the cages, and no tree was found to grow within the group's used core area of NHE-3. However, especially by group J&D only tiny amounts were ingested on those days *C. canadensis* branches were provided within the cages.

Another plant species, which was differently eaten between groups of the same season and of different seasons, was *L. styraciflua* (Fig. B.10).

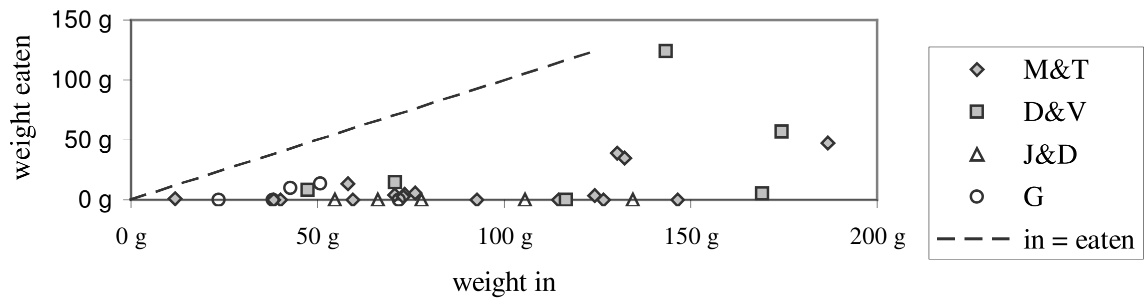


Fig. B.10: Correlation table and diagram between the weight of *Liquidambar styraciflua* leaves put into the cage and the amount ingested by each study group.

Regarding the spring season, despite outdoor access of group M&T, *L. styraciflua* was definitely more eaten by group D&V within the cage, albeit not statistically significant.

However, *L. styraciflua* was regularly eaten during the spring, equally inside the cages and outside in the forest, whereas it was nearly not selected within the cages during the autumn. On the contrary, in autumn it was highly favoured in the forest by group J&D, and comparing the groups with outdoor access even more was selected during the autumn than during the spring session.

With respect to the total amount eaten by group M&T, *L. styraciflua* constituted an average of 2 % (Tab. B.8) and was heavily selected within the forest. Group D&V ingested an average portion of 3 % of *L. styraciflua*, while in the autumn *L. styraciflua* accounted for 9 % within the diet of group J&D and 1 % of Gordian. Nevertheless, the largest portion of *L. styraciflua* referred to bodyweight was eaten by group D&V in spring, although statistically not significant. Statistical significant differences are evidenced in eating *L. styraciflua* between the groups M&T and J&D ($p < 0.01$, U-test, two-sided) and on the same level of likelihood comparing group J&D to Gordian.

It has to be emphasised that a *L. styraciflua* tree was the main tree of residence for group J&D within the forest, an indication of the individual predilection for this tree species. However, at this point, it cannot definitely be concluded why *L. styraciflua* leaves were much more eaten in the forest than within the cages during the autumn session. Similar to *A. julibrissin*, the age of leaves might be an important aspect, which could be selected to their liking in the forest in contrast to caged conditions. On the other hand, if already enough *L. styraciflua* leaves were consumed in the forest, there was no need for group J&D to select the leaves within the cage. Hence, reasons for eating *L. styraciflua* differently are to be searched in its supply, presumably in the age of the leaves available and in personal preferences.

R. pseudoacacia trees were growing in both natural habitat enclosures, but only few leaves were eaten within the forest during both seasons. In both, the spring and the autumn season, it was seldom brought into the cages, but once available there, it was more eaten during the spring than during the autumn (Fig. B.11). The only statistically significant correlation between leaves brought into the cage and the quantity eaten is figured out for group M&T. However, in spring both groups ingested about 40 % of the leaves provided within the cages, whereas in the autumn only 8 % were eaten by Gordian and nothing by group J&D. Group J&D ate *R. pseudoacacia* leaves only in the forest.

group	r_s	prob. of error	n
M&T	0.8	$P < 0.05$	4

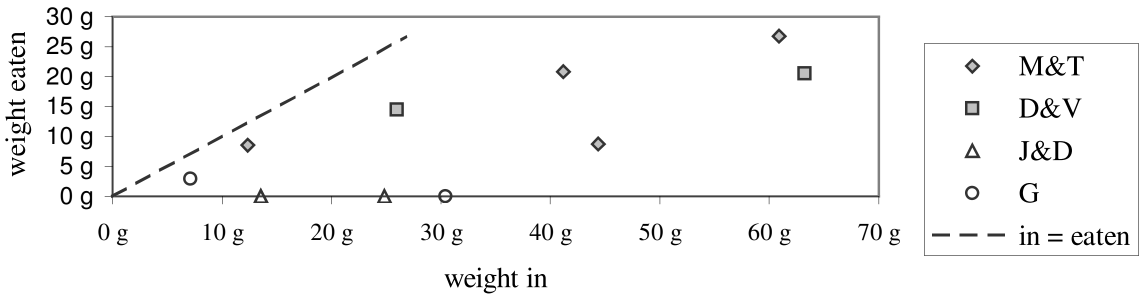


Fig. B.11: Correlation table and diagram between the weight of *Robinia pseudoacacia* leaves put into the cage and the amount ingested by each study group.

The situation for *L. tulipifera* (not depicted as diagram) is similarly complicated as for *A. rubrum*, but *L. tulipifera* is more difficult to evaluate in the spring. *L. tulipifera* was only once brought into the cage of group M&T and never to group D&V. Hence, in spring eating *L. tulipifera* can only be evaluated for group M&T, and it could be best selected within the forest. While nearly all of the leaves offered within the cage was eaten by group M&T, only once it was eaten in substantial amounts within the forest. On the contrary, in the autumn group J&D typically ingested every day large amounts of *L. tulipifera* leaves in the forest, but not within the cage. Gordian's food choice only relied on the amount offered within the cage. It remains to be hypothesised, if Gordian ate less leaves than group J&D due to the age of leaves offered or if he had other reasons for not selecting *L. tulipifera* the same way as group J&D.
However, like *L. styraciflua* leaves, a percentage of 9 % was eaten of *L. tulipifera* by group J&D, while for Gordian it amounted to 2 % (Tab. B.8). In the forest, *L. tulipifera* leaves were generally more eaten by group J&D than by group M&T. Concluded from the set of available data, *L. tulipifera* was obviously more eaten during the autumn session than during the spring, but this may result from the different feeding conditions between the groups.

P. glabra leaves (neither depicted as diagram) were heavily eaten in the autumn when provided. As these leaves are typically provided only during the autumn and the winter season, nothing can be said about other seasons. It has to be noticed that *P. glabra* builds a big portion of the winter food since the desired reddish leaves at the tip of the stems are available throughout winter time (Haring, pers. comm.). Referred to bodyweight, *P. glabra* leaves can be classified as preferred food item. It was only once provided to each group during the autumn session, but then heavily eaten by both groups. The amount eaten by Gordian even yielded a percentage of 1 % of the total food eaten (Tab. B.8).

For the groups M&T and J&D, the remaining leaf species within the diet were mainly shared among those species, which were vigorously selected in the forest, but not presented in the cages such as leaves of *Morus rubra*, *Vitis* sp., and *Oxydendrum arboreum* (Tab. B.5 and Tab. B.6).

Comparing the feeding behaviour seen within the forest during the autumn and the spring season, it is remarkable that the amount eaten by group J&D in NHE-3 is about thrice as much as that eaten by group M&T in NHE-1, while the time of group J&D spent in the forest was nearly double the amount of time as granted to group M&T. Whereas in the autumn approximately 60 % of the leaves were selected in the forest, only 17 % of leaves were selected from the forest by the study group in spring. It is obvious that group J&D did not spend much time on tasting any species, presumably because they already knew well their forested territory.

Furthermore, it has to be kept in mind that during the autumn session typically only 3 different leaf species were provided per day within the cages. Hence, except for *R. copallina* and *A. julibrissin*, the feeding pattern for most plant species can best be evaluated considering the leaf selection of group J&D within the forest. Observations in the forest are vital for the evaluation of plant species and plant parts. Finally, it can be concluded that holding conditions, food supply and habituation to food resources have an enormous impact on food choice, but despite these factors the portion of leaves, and especially two selected leaf species, play a vital role within the sifakas' overall diet.

4 Discussion

Different conditions of captive animal maintenance and small group sizes[1] allowed for an intense data collection within this study. Although free ranging forested animals could have been problematic, these animals could be closely observed and were followed at all times with food intake noted that was based on weight. Although feeding behaviour on caged animals typically are based on weight, the instigation of the estimation method is new for animals ranging in the forest.

Although the methods described by Altmann (1974), which are well established in field studies in order to determine total food intake based on time spent feeding (Richard, 1977 and 1978a; Powzyk, 1997) instead on weight, this method of time measurement does not account for discrepancies between time needed and amount eaten. As already discussed by Richard (1978a) and corroborated by observations of the feeding bouts in this study (App. I.2), animals may need only a short time to ingest a large portion of e.g. a desired leaf species, but utilise a longer feeding time to gnaw at wood or on dry needles. Conversely, in the case of nutrients garnered from nuts, nutrient gains parallel the time needed but then shows an anti-parallel trend with the amount of nuts ingested. In other words, animals needed more time to ingest small amounts of Brazil nuts, but nutrient and energy gain of this food item is high due to its high fat content. Another point is that feeding speed depends upon how hungry the animal is. The observation that animals slowed down their feeding rate during a feeding bout after having consumed most of the food eaten is corroborated by similar descriptions of free-ranging animals (Richard, 1978a). In captivity, most food was eaten during the first ten to twenty minutes, which were followed by reduced feeding rates, and essentially preferred food items which were nearly exhausted.

Of course, the estimation method instigated in this study bears inaccuracies regarding the assignment of an exact weight to an amount (or portion) of food specimen eaten. For example, when focal animals were feeding in the forest, an animal may be partly hidden by a branch while feeding. Often it was difficult to make out the type of leaf species that was being consumed when the animals were sitting or hanging in a tree while reaching over to eat from a nearby tree in an overlapping canopy. There were also difficulties in monitoring caged animals when it proved difficult to count food pieces and parts consumed when animals buried their mouths in the feeding bowls. However, when scoring selected plant species and estimating their corresponding weights in the cages provided vital information on the animals' feeding patterns, especially when recording amounts in the forest when weighing of food consumed was impractical.

Observational data corroborated the importance of a food species for the animals' diet and completed their overall food preferences. For example, sometimes during the spring season it could happen that less *Rhus copallina* and *Albizia julibrissin* leaves were provided within the cages than were obviously desired by the animals. Typically these leaves were initially consumed during the main feeding bouts yet the animals were observed to search for these items for a protracted period of time from the leaf bunches, which emphasises their importance within the animals' diet. The mere weights eaten were in these cases less meaningful inasmuch that the desire for the food exceeded the quantity of food provided.

Hence, the combination of the weighing method, the estimation method and additional observational data allowed for a determination of the daily food eaten and helped to decipher general, individual and seasonal feeding patterns.

[1] Normally, groups of *P. verreauxi* consist of 2-13 animals (Richard *et al.*, 1991; 1993).

As in human's nutrition, all weights ingested are referred to "fresh weight". To determine the nutritional and energy values of the fruits and vegetable portions (Souci *et al.*, 1994) with respect to plant specimens ingested in the forest, I preferred to describe all foods to the "fresh weight" category instead of "dry weights" which is typically used in animal nutrition (detailed investigations see chapter C.I).

The advantage of this study design is that data collection can be applied to field-like conditions. Methods used are relatively easy to perform and do not need any adjustment to the animals in regard to a changed feeding regime, holding conditions, or utilisation of equipment. This latter point was vital, as the DULC research management would not have approved any alterations. Nevertheless, this was disadvantageous insofar, as not even browse, especially the most preferred species *R. copallina* and *A. julibrissin*, could be given *ad libitum*, to determine the upper level of desired intake. Therefore it was not possible to feed the same leaf species in the cages on a daily basis during both seasons, so that the forest became even more important to evaluate leaf selection. Observations in the forest virtually completed the overall image of the lemurs' dietary preferences especially of those plant species which were not or not regularly given in the cages during either season. For instance, *Acer rubrum* was regularly offered in the spring but not given in the autumn. Fortunately, it grew wild in both natural habitat enclosures and I was able to compare both seasons to one another. Furthermore, the more natural diet during the spring season was changed in the autumn to a diet superabundant in chow. The data records reflected the feeding regime and holding conditions at the DULC during both study periods. It is notable that both sets of data, which were gained day by day, allowed for an intense insight into the feeding behaviour of the focal sifakas, albeit only relatively short moments within their life were documented. Obviously, animals coped with both diets offered and the analyses of nutritional and energy values will reveal if they held nutritional and energy contents constant (see chapter C.I).

Yet food supply does influence consumption and the phenomenon of an apparent lower food intake by animals during the autumn compared to the spring is obviously due to the larger portion of chow offered. Therefore groups in spring consumed more fruits and vegetables combined with a larger portion of leaves (Fig. B.3, p. 40; Fig. B.5, p. 42) than foods eaten in the autumn. So, the apparent dependency on the animal's age of the total weight ingested is more reflected by the portion of fruits and vegetables plus chow than by the amount of leaves. Despite a different food supply during the spring and autumn, at least one third (31 % and 33 %, respectively) of the total fresh foods were allotted to the animals as a portion of leaves within the caged groups (Fig. B.3, p. 40), which is in agreement with findings of Lopes Santini (1992) on *P. v. coronatus* at the Zoological Park of Paris and a study performed by Campbell *et al.* (2001) at Duke on *P. v. coquereli*. It is notable that both groups with outdoor access within this study raised the percentage of leaves within their total diets to 38 % and 45 %, respectively, which emphasises the importance of the leaves within the sifakas' diet.

A comparison with previous observations in captivity and on free-ranging *P. verreauxi* in the wild corroborate that food preferences are dependent on factors such as different seasons, individual food preferences and general food availability. In this study, the availability of seasonal occurring plant parts such as flowers or seeds was low. However, animals with outdoor access received a natural-like diet with respect to the availability of different leaf species, albeit their favourites *R. copallina* and *A. julibrissin* were only provided within the cages. Therefore, the feeding behaviour was only referred to plant species and plant parts the focal lemurs found in their environment during the observation periods.

Based on a different degree of being habituated to the forested enclosures, a lot of plants were tasted and proved positive or rejected by the group M&T, while this behaviour was not observed for the group J&D. The behaviour of tasting small amounts of unknown food sources is in accordance with the behaviour postulated by Freeland and Janzen (1974).

Accordingly, novel foods were treated with caution and sensory capabilities as well as memory may play a vital role.

Although it can be presumed that the olfactory sense is more developed in prosimians than in higher primates (Schilling, 1979), and smelling, licking, tasting are important capabilities which are prerequisites to survive in the forest, nearly nothing is known about the exact mechanism how animals decide if a plant species is palatable and beneficial or detrimental. However, this interesting aspect lies beyond the scope of this study and needs further investigation.

During the winter time at the DULC, the sifakas received approximately 90 % *R. copallina* leaves and 10 % *A. julibrissin* leaves, which were frozen in September. Young, fresh leaves of *Photinia glabra* supplemented the winter diet as “fresh” leaves and are available from autumn through the winter time. From April on animals received fresh leaves by providing the species of the browse mentioned. Therefore, *R. copallina* and *A. julibrissin* could be consumed all year round (Haring, pers. comm.). The clear preference for *R. copallina* leaves of Coquerel’s sifakas was already mentioned by Haring (1988). The importance of *R. copallina* leaves for *Propithecus* species at Duke was emphasised by the behaviour of two *P. tattersalli*, who quarrelled for a bifurcate branch of *R. copallina*, each sifaka pulling at one side, until the branch broke into two pieces.

With respect to the seasonal availability of food items and food selection at the DULC, Haring (1988) reported that *P. verreauxi* spent a lot of time in *Carpinus caroliniana* and *Acer rubrum* with ripened seeds. During the observation periods of this study, seed ingestion was not observed except for *Cercis canadensis*, due to the seasonal availability of these food items. While *A. rubrum* leaves were eaten, the focal lemurs only tasted the leaves of *C. caroliniana* within this study. Mango leaves, which are reported to have supplemented winter diet for some years to overcome the seasonal crisis in the animal’s condition and which were therefore imported weekly from Florida and frozen (Haring, 1988), were not ingested by the focal lemurs of this study. The focal lemurs only liked the mango fruits. However, *Mangifera indica* is reported to be ingested by *P. tattersalli* in Madagascar (Meyers, 1993). *Liquidambar styraciflua* leaves are reported to be an important food item for the Coquerel’s sifakas at the DULC, which agrees well with own observations. Fresh *L. styraciflua* leaves are available during most time of the year and were frozen to be available during the winter time (Haring, 1988). *Robinia pseudoacacia* leaves were eaten as less preferred food item within this study, albeit they were highly selected by captive *P. v. coronatus* at the Zoological Park of Paris (Lopes Santini, 1992), which corroborates the variability in food preferences among related *Propithecus* subspecies (Richard, 1978b).

According to Richard (1977, 1978a, b), a high proportion of time was spent eating bark or dead wood at certain times of the year by either *P. verreauxi* subspecies. *P. v. coquereli* was seen to eat bark to the exclusion of dead wood, while *P. v. verreauxi* was never seen to eat dead wood. Similarly, in captivity Coquerel’s sifakas ate dry needles of *Pinus taeda* trees and ingested occasionally bark or wood.

Some of the plant genera growing in the North Carolinian forest were reported to occur also within the Malagasy forest. According to Richard (1977, 1978a), *P. v. verreauxi* fed on *Diospyros humbertiana*, and on *Mimosa* and *Albizia* species for considerable amounts of time, which ranked within the most eaten twelve Malagasy species. During Richard’s study, one *Mimosa* species (Mimosaceae) was chosen by *P. v. verreauxi* to build an important food item throughout the year. The most eaten species belonged to the genus *Mimosa* (Mimosaceae). Studies performed about 20 years later revealed again the obvious importance of *Mimosa* (*Mimosa minitifolia*) and *Diospyros* species for *P. v. verreauxi* living in the arid Didiereaceae forest in the south of Madagascar (Ranarivelo, pers. comm.).

While *P. verreauxi* at Morondava selected the leaves of *Albizia greveana* and *Diospyros aculeata* (Ganzhorn, pers. comm.), *P. tattersalli* was interested in *Albizia gummifera*, *A. boivini*, *A. gaubertiana*, *A. lebbeck*, *Diospyrus lokohensis* and *D. sakalavarum* (Meyers, 1993). *P. d. edwardsi* was reported to be very interested in *Albizia gummifera* (Hemingway, 1998). Different species of the genus *Albizia* were also selected as food source by other primate species like chimpanzees (Ganzhorn, pers. comm.) or red colobus monkeys (Struhsaker, 1978). At the DULC, the leaves of both species, *A. julibrissin* and *Diospyros virginiana*, were selected with seasonal differences (see Tabl. B.7, p. 34). Tree species of the Anacardiaceae were found among the 12 most selected plant species of wild Coquerel's sifakas in Madagascar (Richard, 1978b), while *R. copallina* was the most selected leaf species at the DULC.

In Madagascar, differences in food preferences were also evidenced within neighbouring groups of the same *Propithecus* subspecies (Richard, 1977; 1978a, b). Differences reached from using or rejecting an entire tree species to differences in the extent to which a tree species was used or just to differences in the use of plant parts of the same species. Only eight out of twelve species most commonly eaten by two neighbouring groups of each *Propithecus* subspecies were the same (Richard, 1978a). This seems to resemble the findings on individual food use and different individual food preferences within this study: there was a different utilisation of *Morus rubra* leaves by Marcella and Tiberius, and different amounts were eaten of *A. rubrum* and *C. canadensis* leaves by the groups M&T and D&V. This aspect is mirrored by findings of Lopes Santini (1992) on preferential food use by individuals of captive *P. v. coronatus*.

In Madagascar, seasonal changes markedly influenced the dietary composition and the time spent feeding. All sifakas spent more time feeding in the wet season than in the dry season, while the number of species eaten increased in the dry season (Richard, 1977; 1978a, b). One group of Coquerel's sifakas was seen feeding on 85 different food species, the other on 98. If only food species diversity is considered on which animals fed more than 1 % of total feeding time, 22 different food species were counted with a marked decrease in the wet season. Nine food species were eaten for more than 1 % of the total time spent feeding in both the dry and the wet season out of 99 different food species, which were eaten in either season by at least one of her two study groups (Richard, 1978a, b).

More than 65 % of the total feeding time were spent eating on only 12 different plant species by both subspecies, *P. v. verreauxi* and *P. v. coquereli*, respectively. Almost two-thirds of the total number of food species were only rarely eaten, while only few species were primarily eaten (Richard 1978a, b). However, Richard (1978a) found a large overall long-term stability in food choice throughout the years. Food species chosen and the feeding time allotted to them were found to be much the same, which seems also to be valid for captive conditions (compare Haring, 1988).

Generally, the same pattern of a selective food choice is already well documented for other members of the indriid clade like *P. d. diadema* (Powzyk, 1997), *P. d. edwardsi* (Hemingway, 1996; 1998) and *P. tattersalli* (Meyers, 1993), which also agrees with findings of this study. Despite a large offer of plant species (including species growing in the forest), only some leaf species were heavily eaten. Moreover, Richard (1978a) found two patterns in using plants as food source: both *P. verreauxi* subspecies used respectively one plant species throughout the entire study time, while a second plant constituted the bulk of the group's diet during a short period of time, apart from which it was of little or no importance. Similarly, in captivity *R. copallina* leaves were nearly constantly and heavily eaten throughout both observation periods and irrespective of seasonal influences, while *A. julibrissin* leaves were preferentially selected during the spring season. Both species were selected even by those animals who had spent hours within the forest, where a lot of other tree species had been available.

In contrast to the leaves, a large variety of fruits and vegetables was offered and eaten by the sifakas within this study (see Tab. B.3, p. 19). These findings agree with results found for *P. v. coquereli*, *P. tattersalli* and *P. d. diadema* in a later study at the DULC despite a different study design and a diet even more pronounced in chow (Campbell *et al.*, 2001).

In Madagascar, proportions between different plant parts within the diet of *P. verreauxi* fluctuated seasonally, hence far from being constant (Richard, 1978a, b). Although the overall change between food parts (immature and mature leaves, flowers, fruits and to a minor extent other plant parts) was similar between both *Propithecus* subspecies, important differences emerged. Coquerel's sifakas ate more immature leaves and less fruits and mature leaves than Verreaux's sifakas. However, averaged on the year, time spent feeding was more granted to fruits than to either immature or mature leaves, and only to a lesser extent to flowers and other plant parts within both subspecies. Hence, although *P. verreauxi* is classified as leaf-eating primate (Richard, 1978a, b) the portions between leaves and fruits within their diet were found to be largely fluctuating with seasons and forest phenology, which emphasises the ability of *P. verreauxi* to cope with the challenge of fluctuations in food availability. The availability of appropriate food is an important determinant to survive in nature and determining an animal's ecological niche. In captivity, the portions of fruits and vegetables plus chow were nearly constantly available irrespective of the season and built the largest portion within the daily food of the focal sifakas. The different portions of leaves, fruits and vegetables within the diet of the four study groups (Fig. B.3, p. 40) obviously lie within an acceptable and tolerated diet.

5 Conclusion

The major goal of this chapter is to evaluate food quality and quantity while taking into account holding conditions, individual food preferences and seasonal factors which may influence lemur food choice. The foods available to the study lemurs consisted of a variety of fruits and vegetables, leaf-eater primate chow, together with a selection of leaf species. Noting the individual kg bodyweight of the focal lemurs, the weights of the consumed fruits and vegetables and leaf-eater primate chow, differed substantially between the four sifaka groups and were dependent on the animal's age along with distinct seasonal preferences in food consumption during the spring and the autumn data sessions. Although there was disparate food availability due to the climate seasons, overall less leaves were consumed in the autumn compared to the spring, albeit their percentage within the sifakas' diet remained rather constant. Despite different feeding and captive holding conditions with the focal lemurs which also varied in age, at least one third of the entire food intake was allotted to leaves, with an average of 32 % (m/m) noted in the diet of the caged animals, and an average of 42 % (m/m) within the diet of those groups with outdoor access.

From the portion of the self-selected leaf species, two leaf species were revealed to be essential for the animal's preferred diet and observations also revealed a distinct pattern of leaf choice. *Rhus copallina* (sumac) and *Albizia julibrissin* (mimosa) leaves were vigorously chosen by all lemur study groups irrespective of their holding conditions, independent of the animal's age, but with a distinct seasonal difference in selecting *A. julibrissin* leaves. While *R. copallina* leaves were eaten regularly in both seasons, *A. julibrissin* leaves were eaten significantly less in the autumn. Apart from some individual leaf preferences, the bulk of other leaf species consumed seemed to be of minor importance for the sifakas. The most preferred plant species, *R. copallina* and *A. julibrissin* deserve further investigation with respect to primary, and secondary plant compounds.

C Chemical investigations on the major food and drug resources

C.I Nutritional and energy values of foods selected by *P. v. coquereli*

1 Introduction

The main food resources in the diet of captive Coquerel's sifakas are leaves from selected tree species, a variety of fruits and vegetables together with a commercial primate chow. Generally, major nutrients gained from these food specimens are structural and non-structural carbohydrates, proteins and fats. Of course, the capability to digest these food components to gain nutrients and energy, especially with respect to fibre sources, depends largely on an animal's gastrointestinal adaptations together with the microbial flora, which in turn determines the animal's food choice and feeding strategies (Hofmann, 1983; Garber, 1987; Langer, 1986; Langer, 1988; Stevens, 1988; Hofmann, 1991). For folivorous midgut-fermenting[1] mammals, fibre sources contribute both to the nutrient and energy supply, but also influence the digestion and digestibility of other dietary components. In addition, they are involved in processes which maintain a healthy gut flora (Cook and Sellin, 1998; Onderdonk, 1998). Hence, dietary limitations imposed to the animal by its digestive capacities are normally reflected by feeding selectivity and food quality.

Although the Coquerel's sifakas in this study live in captivity and do not suffer from restrictions in nutrient and energy supply, they exhibit a strong feeding selectivity and diets in previous years proved to be somewhat detrimental to the animal's health and well-being (Haring, 1988).

In the following investigation, the composition of primary food constituents in the diet of *P. v. coquereli* and their nutritional benefit to the animals is to be assessed. Major questions to be answered are: What is the food composition of the present diet like with respect to primary food compounds? In what way do individual food resources contribute to nutrient and energy supply? With respect to seasonal changes in primary leaf compounds, how do food preferences differ in the spring and autumn as to portions of fruits / vegetables and chow? And during these seasonal changes is the composition of primary food constituents held constant, or is the nutrient composition altered? Overall, what is the impact of heightened leaf consumption on the health of the sifakas, and why did the focal animals exhibit such selectivity in their leaf consumption?

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[1] previously known as hindgut-fermenting mammals

2 Materials and Methods

2.1 Plant material: origin and identification

All plant material used for chemical analysis and pharmacological tests was collected near the Duke Lemur Center, North Carolina, USA. It was collected throughout the study in May 1994 and October 1997. Leaves were air-dried while still attached to their branches (and shielded from direct sunlight), and were later stripped from their branches and stored in zap-lock bags to be brought back to Germany (Tab. C.1).

Additional plant material was collected in July 1996, of the two major food plants *R. copallina* and *A. julibrissin* by members of the Duke University Botany Department and were dried and sent to Germany (about 1 kg dried leaves each).

In 1994 only leaf species given as browse were collected and dried, whereupon the first chemical analyses started with these initial specimens. During the follow-up study in the autumn of 1997, additional species were collected (Tab. C.1), which were either provided as browse or selected from the forest by the study animals.

All plant material collected was first identified by means of the "Audubon Society Field Guide to North American Trees" (Little, 1993), and later verified by Prof. Dr. Robert L. Wilbur at the Duke University Botany Department.

Tab. C.1: Review on plant species and time of collection.

plant species	collected in		
Albizia julibrissin	May 94	July 96	October 97
Rhus copallina	May 94	July 96	October 97
Cercis canadensis	May 94		October 97
Robinia pseudoacacia	May 94		October 97
Acer rubrum	May 94		October 97
Liquidambar styraciflua	May 94		October 97
Liriodendron tulipifera			October 97
Photinia glabra			October 97
Vitis rotundifolia			October 97

The selection of plant species was based on the feeding behaviour of the sifakas observed during the months of May 1994 and October 1997. Typically, only the leaf blades or leaf blade parts were ingested while the stalks were consumed occasionally by some study individuals. Therefore, leaf stalks were generally removed from the leaf material before starting any analyses. Leaflets of stalkless species such as *A. julibrissin* and *R. copallina* were stripped off the axis, similar to the technique observed by the animals. The material harvested were comprised primarily of the plant species fed as browse to the caged lemurs and represent those species which had been selected by the sifakas before the onset of this study.

To harvest the leaf material, branches and twigs were cut including upper immature, younger leaves and lower intermediate and mature leaves. All leaves of that branch were stripped off and mixed for subsequent analyses. Only for *A. julibrissin* leaves was it necessary to distinguish in spring the immature leafbuds at tips of the bipinnately compound leaves and lower young but mature leaflets, while in autumn a distinction between older, very mature and younger mature leaflets, was guided by size, colour and texture of the leaflets. This discrimination in turn was based upon the feeding behaviour exhibited by the sifakas.

2.2 Preparation of the dried plant material for chemical investigations

According to the feeding behaviour of the sifakas, the air-dried leaves were stripped off the stems and twigs so that only the leaf blades remained. Depending on the leaf species, leafstalks were only tolerated to the extent they have been ingested by the sifakas. Afterwards, the dried leaves were ground by different methods:

Smooth leaf species containing very fibrous tissue, such as *A. julibrissin*, *C. canadensis* and *R. pseudoacacia*, were first crushed by a mill (Braun®, or most often by Moulinex®) and then ground by means of a mortar and pistil. In contrast, the tougher leaves such as *R. copallina* and *L. styraciflua*, were already sufficiently powdered by the latter method. To get a uniform particle size of the powdered leaf material, it was finally standardised by using sieve No. IV (DAB 7, corresponding to 0.80 mm DIN 4188). Standardisation excluded the tougher and more voluminous and fibrous parts of the leaf texture and yielded particle sizes appropriate for the different analytical investigations.

Leaves of *P. glabra* were quite resistant to most grinding trials so that only few amounts of powdered plant material could be provided for assays where fine and uniform leaf material was needed. Hence, the number of analyses was limited for this species.

2.3 Nutritional and energy values of the food

The nutritional values of the sifakas' diet are determined by means of two different sets of data. Nutritional values of the leaves were determined experimentally according to methods of the Weender analyses (Naumann and Bassler, 1976-1997; Meyer *et al.*, 1993). Nutritional values of the large portion of fruits and vegetables were taken from the nutrition tables of Souci *et al.* (1994).

Food resources such as leaf-eater primate chow (No. 5672, Purina Mills, Inc. St. Louis, MO) and chick peas were calculated according to information given by the respective production companies. Data missing on the primate chow were completed by methods of the Weender analyses, while data missing on chick peas were completed by means of the nutrition tables (Souci *et al.*, 1994).

Nutritional values according to Souci *et al.* (1994), are adjusted to human's food use, and the classification of analytical data is based on the digestibility of the human's digestive system. In the nutrition tables, primary compounds are referred to fresh food weight and all food specimens are referred by their respective edible portion, that is raw products as purchased on the European market minus waste products (e.g. peels, kernels, shells) to be edible for humans.

As there was no fundamental difference in processing fruits and vegetables prior to consumption between humans and sifakas with respect to the species of fruits and vegetables offered (chapter B), the large portion of fruits and vegetables was evaluated by means of the nutrition tables of Souci *et al.* (1994). In consequence, primary compounds analysed for the air-dried leaves had to be converted to fresh weight since all food intake is based on fresh food weight.

While the content of food constituents is normally given in g / kg in animal nutrition (App. II.1), it was preferred to use g / 100 g food specimen in agreement with Souci *et al.* (1994) in human nutrition.

Energy values of the various fruits and vegetables were also taken from the nutrition tables. Energy values of the leaves and all other food sources not included in the tables were calculated by means of the respective energy factors for available carbohydrates, proteins and fat as given by Souci *et al.* (1994) and listed in Tab. C.2.

In this study two different models were used to calculate minimum and maximum energy gain from the leaves in the diet of the sifakas: either the fraction of fibres was not regarded, or it was calculated by means of the respective factor taken from Tab. C.2.

Tab. C.2: Gross energy (Meyer *et al.*,1993; Biesalski *et al.*, 1995), metabolisable (physiological) energy (Biesalski *et al.*, 1995), and energy factors used in this work (Souci *et al.*, 1994) of different nutrients.

nutrients	**energy in kJ / g[1]**		
	gross	**metabolisable**	**factor**
carbohydrates	17.2	17.2	17
protein	23.8-24.2	17.6	17
fat	36.6-39.8	38.9	37
organic acids			13
fibre	17.2-22.2		(0-)17[a]
nitrogen free extract	17.0-17.5		
urea	10.7		
uric acid	11.5		
acetic acid	14.6		
propionic acid	20.8		
butyric acid	24.9		

[a] For maximal energy intake the content of fibre and the organic rest of the leaves are calculated by factor 17.

While gross energy is the energy content of nutrients determined as heat by means of a bomb calorimeter, the metabolisable (physiological) energy reflects the actual portion of energy gained by the organism. Main energy losses are due to faecal output, urine and the production of gases (Tab. C.2). In contrast to the metabolisable energy, the digestible energy is defined as gross energy intake minus faecal energy losses. Without further correction for faecal energy of endogenous origin, this digestibility is defined as “apparent digestibility”.

Until now the use of nutrition tables based on human nutrition was well established as the source of data in primate husbandry (Müller and Schildger, 1992) and is used to calculate metabolisable energy obtained from the food (Conklin and Wrangham, 1994).

Determination of primary compounds in the leaves (Weender methods)

The following “Weender methods of analyses” (Naumann and Bassler, 1976-1997; Meyer *et al.*, 1993) were applied to determine the nutritional values of the leaves selected by the sifakas. Analyses were performed on the air-dried plant material at the laboratory of Prof. Dr. M. Coenen (Tierärztliche Hochschule, Hanover).

While detailed analyses according to methods listed were only carried out for *R. copallina* and *A. julibrissin*, for all other leaf species collected dry substance and crude ash were determined, respectively. The respective contents of crude protein, available carbohydrates (simple saccharides and starch) and crude fibre were calculated by averaging the respective data examined for *R. copallina* and *A. julibrissin* of either season. Normally, analyses were performed in duplicate (App. II.1).

Determination of dry substance and crude water

Approximately 3 g of air-dried and ground plant material are dried in an oven overnight at 105 °C (according to Meyer *et al.* (1993) for four hours at 103 °C), that is to constancy of weight.

[1] Relation between calorie and Joule: 1 cal = 4.184 J

The difference in weight after drying is predominantly due to the loss of water, but includes also all other substances volatile at the temperature of drying, e.g. volatile fatty acids, essential oils, alcohol etc. The loss of weight is defined as crude water.
dry substance = air-dried plant material – crude water

Comment:
While the determination of "weight loss by drying" according to DAB 7 and 8 is a conventional method for drugs and extracts, and is used for quality control, the determination of the dry substance according to Weender analyses is performed to constancy of weight. All subsequent analyses of the Weender analyses are referred to dry substance of each plant specimen.

Crude ash
Plant material is put into a muffle furnace at 600 °C (550 °C according to Meyer *et al.*, 1993) for six hours, until constancy of weight. The residue constitutes the crude ash containing minerals, trace elements and other anorganic compounds as silicates.

Crude fibre
1 g plant material is refluxed for 30 minutes in 1.25 % aqueous sulphuric acid, and after removal of the acidic solution, it is refluxed in 1.25 % aqueous sodium hydroxide and rinsed with boiled water. The residue is dried overnight at 105 °C, cooled down in an exsiccator and weighed. Finally, that residue is muffled in a furnace at 500 °C for about two hours.

Thereby, crude fibre content is determined by weight difference between the residue after drying at 105 °C and the residue after muffling referred to the input of plant material.

Crude fibre means the fat- and ash-free residue of the plant material that is insoluble in acidic and basic solution. It contains insoluble portions of cellulose, hemicellulose (pentosanes, hexosanes), pectin, lignin and other parts of the plant cell wall as waxes (cutin, suberin).

Crude protein
The determination of the crude protein according to the "Weender analyses" is based on the Kjeldahl method (Ph.Eur. I). The plant material is decomposed and oxidised by means of conc. sulphuric acid, copper sulphate and conc. H_2O_2. The latter reagents serve as catalysts (Roth and Blaschke, 1981). Hence, all nitrogen is converted into a corresponding amount of $(NH_4)_2SO_4$. A solution of sodium hydroxide is added in surplus and the volatile ammoniac is distilled according to Ph.Eur. I into an acidic solution, here 2 % boric acid, which is more likely to serve as buffer. Unlike Ph.Eur. I, which uses a back-titration of the surplus of acidic solution, the content of NH_4OH is here directly determined by titration with 0.3 M HCl.

Comment:
Protein contains an average of 16 % nitrogen. (Crude protein = N x 6.25).
Disadvantage of the Kjeldahl method is, that also nitrogen containing compounds of non protein nature are included, e.g. acid amides, amines, free amino acids, ammonium salts, alkaloids. Azo-groups cannot be determined by this method as molecular nitrogen is formed. Pyridine- and chinoline- derivatives are difficult to decompose under Kjeldahl conditions and are therefore difficult to quantify (Roth and Blaschke, 1981).
Yet, with respect to the well adapted gut system of the sifakas which is probably able to use also other sources of nitrogen apart from proteins, the determination of nitrogen according to Kjeldahl is an appropriate method. However, compounds that may fail to decompose or cannot be determined by this method, yet are not expected to be found in prodigious amounts within the food plants.

Determination of simple (mono- and di-) saccharides
The content of mono- and disaccharides (except for lactose and maltose) is determined by iodometric titration following acidic hydrolysis. This procedure determines glucose, which is referred to sucrose when calculating the content of simple saccharides.

The dried plant material is dispersed in 40 % aqueous ethanol to solve selectively mono- and disaccharides, while starch and related compounds of higher molecular weight are not solved under these conditions. Carrez I reagent ($ZnAc_2$ in aqueous acetic acid) and Carrez II reagent (aqueous solution of $K_4(Fe(CN)_6 \times 3 H_2O)$ are added to precipitate proteins as well as other complexing compounds. (Carrez I precipitates proteins while Carrez II enhances precipitation.)

The solution is then filtered, neutralised with hydrochloric acid against methylorange (indicator) and hydrolysed by means of 0.1 M HCl for 30 min. at 100 °C to yield glucose and fructose.

After neutralising the hydrolysed solution with a corresponding quantity of 0.1 M NaOH, Luff-Schoorl reagent (copper II solution) is added to oxidise glucose to gluconic acid by transferring two electrons.

To quantify the gluconic acid yielded, potassium iodide is added, which reduces again gluconic acid to glucose (and the residual Cu^{2+} to Cu^{+}) while self being oxidised to iodine (I_2). Iodine is titrated with thiosulphate against starch.

Determination of starch
The content of starch is determined by polarimetry, which is performed twice.

First, the entire solution of carbohydrates is hydrolysed for 15 min. at 100 °C in 1.12 % aqueous hydrochloric acid, clarified by Carrez I and II reagents and filtered to be determined by polarimetry. Second, the optical activity is determined after selective extraction of mono- and disaccharides with 40 % ethanol and subsequent treatment as for the first determination. The entire optical activity is diminished by the optical rotation of carbohydrates soluble in 40 % ethanol.

Definition of terms used in this study

Water
The water content of the fresh leaf material was determined before and after air-drying and was averaged irrespective of species and season and set constant at 82 % (weight), which is in accordance with the literature (Lopes Santini, 1992).

Carbohydrates
Carbohydrates according to the nutrition tables ("available carbohydrates", (Souci *et al.*, 1994)) include all mono-, oligo- and polysaccharides (e.g. glucose, fructose, sucrose, lactose, maltose, dextrin, starch) and sugar alcohols (sorbitol, xylitol, glycerol) digestible by the human digestive tract.

The determination of carbohydrates according to Weender analyses comprises simple saccharides (mono- and disaccharides except for lactose and maltose) and starch. The contents of both were added to be treated in this study as non-structural carbohydrates like the "available carbohydrates" of the nutrition tables.

Comment:
The content of non-structural carbohydrates of the leaves contains less components than those analysed for fruits and vegetables according to Souci *et al.* (1994).

Fibres
The fraction of dietary fibres builds a rather heterogeneous group of compounds, raising different problems regarding their determination. Generally, the total fibre fraction consists of two main types: the water-insoluble and water-soluble fibres. While the water-insoluble fibres predominantly contain cell wall structures like cellulose, hemicellulose (polysaccharide type) and lignin (polyphenolic compounds), the water-soluble fibres comprise cell wall structures such as water-soluble hemicellulose and pectins together with cell products such as gum and

mucilage, which have gelling properties when soaked in water. Values for the nutrition tables (Souci *et al.*, 1994) were either determined by the difference method, or by enzymatic methods to identify both: the contents of the water-insoluble and water-soluble fibre types which then yield the total dietary fibres.

Calculation by difference method:
total dietary fibres = 100 – (water + protein + fat + minerals + available carbohydrates)

It is noteworthy to mention that the sum of all food constituents may slightly differ from 100 %, depending on the determination of the contents of carbohydrates and fibres (Souci *et al.*,1994).

Fibres according to Weender analyses ("crude fibre") contain predominantly water-insoluble fibre types with portions of cellulose, hemicellulose, pectins, lignin and other parts of the plant cell wall as waxes (cutin, suberin).

Comment:
Available carbohydrate values characterise types of compounds which are digestible by the human's digestive enzymes in contrast to the fraction of dietary fibres, containing all substances of high-molecular weight which cannot be split up by enzymes of the human digestive system, but rely on microbial symbionts for degradation. However, division based on human's digestibility basically coincides with a division based on chemical structures, that is the non-structural, available carbohydrates ("carbohydrates") and cell wall structures like structural carbohydrates and lignin ("fibres").

Protein
The term "crude protein" according to Weender analyses is termed protein in accordance with Souci *et al.* (1994).

Fat
In the analysis of leaves, the portion of fat is presumed to not exceed more than 1 % of fresh leaf weight (Rust, pers. comm.), which is in accordance with literature (Lopes Santini, 1992).

Minerals
Crude ash values for leaves are determined according to Weender methods, and is used in a similar manner as the value of minerals determined for fruits and vegetables (Souci *et al.*, 1994).

Organic rest
In this study all remaining compounds not determined for the leaves appear in the "organic rest", which is calculated by the difference method:

organic rest = 100 – (protein + fat + fibres + ash + starch + simple saccharides).

It contains mainly water-soluble fibres and carbohydrates not determined in either the crude fibre fraction, or in the fraction of soluble carbohydrates.

The content of "available" organic acids like lactic acid, citric acid and malic acid, which are analysed for fruits and vegetables (Souci *et al.*, 1994), are not investigated for the leaves and appear within the "organic rest".

3 Results

3.1 Food composition and nutritional values

The nutritional values are used to assess the importance of the main food resources - leaves, fruits & vegetables, and chow - and their contribution to animal's diet with special respect to the leaves.

The nutritional values of the leaves according to Weender methods are listed in App. II.1, while values of all food species referred to fresh weight are presented in Tab. C.3.

The evaluation of the primary compounds of the leaves reveals some prominent tendencies during the course of a year. To complete the insight into seasonal changes, *R. copallina* and *A. julibrissin* leaves harvested in July (which has not been observed) and older autumn leaves of *A. julibrissin* (not eaten) were included in the analyses (App. II.1), but excluded when the feeding behaviour was examined (Tab. C.3).

While the protein level of *R. copallina* and *A. julibrissin* leaves is more likely to decrease during the year, the fibre content increases. This is in accordance with the general expectation that mature leaves are more fibrous and contain less protein than immature leaves (Milton, 1979; Waterman, 1984; Powzyk, 1997). The decrease in protein and increase in water-insoluble fibres was also corroborated by a later study at Duke on mixed browse species (Campbell *et al.*, 2001). However, old leaves of *A. julibrissin* harvested in autumn, failed to follow that trend. Older autumn leaves from *A. julibrissin* trees, were found to be less fibrous than younger leaves cut in October, although only the younger leaves if any were eaten during that time of year by the sifakas (see results chapter B). Protein values decreased during the year, and surprisingly the younger October leaves contained less protein than older October leaves (App. II.1).

While animals strongly discriminated between immature and mature leaves of *A. julibrissin*, *R. copallina* leaves were eaten in autumn with nearly the same intensity as in the spring. Gordian ate an overwhelming portion of *R. copallina* in the autumn. Nevertheless, it is remarkable that *A. julibrissin* leaves exhibit very high protein levels in all seasons.

Starch contents typically decreased for *A. julibrissin* yet were increasing for *R. copallina* from spring to autumn. However, this tendency was not entirely consistent. While levels of simple saccharides are again somewhat inconsistent for *A. julibrissin* leaves, levels of mono- and disaccharides increase for *R. copallina* leaves during the year. Again, old *A. julibrissin* leaves collected during the autumn do not fit expectations.

Except for *R. copallina* and *A. julibrissin*, detailed nutritional values on the contents of protein, carbohydrates and fibres were calculated by averaging the respective data examined for *R. copallina* and *A. julibrissin* of either season. (Details see App. II.1). To calculate values for the leaves in autumn, only younger leaves of *A. julibrissin* were taken into account.

When comparing the nutritional values of fruits & vegetables, chow and leaves to one another, some major tendencies can be elicited.

In regard to the protein content of the different food sources, it is striking to note that the commercially designed leaf-eater primate chow contains the largest portion of protein, due in fact to the chow being in a pelleted form with a low water content. Brazil nuts also have a high protein value, and overall a high nutrient density. Furthermore, protein contents of leaves are generally high and range within upper limits determined for vegetables. In contrast, low protein values typically occur in fruits.

Tab. C.3: Average nutritional values in gram per 100 g leaf material, edible portion of fruits & vegetables, and chow, respectively. All values are referred to fresh weight of food specimens.

species		water	protein	fat	carbo-hydrates	fibres	organic rest[a]	minerals / ash
leaves								
A. julibrissin	May	82.00	6.12	1.00	1.60	2.02	6.23	1.03
A. julibrissin	Oct.	82.00	3.87	1.00	1.21	2.96	7.80	1.15
R. copallina	May	82.00	3.13	1.00	1.19	1.57	10.48	0.62
R. copallina	Oct.	82.00	2.07	1.00	2.40	2.53	9.45	0.55
L. styraciflua	May	82.00	4.62	1.00	1.40	1.80	8.37	0.81
L. styraciflua	Oct.	82.00	2.97	1.00	1.80	2.75	8.10	1.38
C. canadensis	May	82.00	4.62	1.00	1.40	1.80	8.18	1.00
C. canadensis	Oct.	82.00	2.97	1.00	1.80	2.75	8.14	1.34
R. pseudoacacia	May	82.00	4.62	1.00	1.40	1.80	8.06	1.12
R. pseudoacacia	Oct.	82.00	2.97	1.00	1.80	2.75	8.37	1.11
A. rubrum	May	82.00	4.62	1.00	1.40	1.80	8.53	0.66
A. rubrum	Oct.	82.00	2.97	1.00	1.80	2.75	8.61	0.87
P. glabra	Oct.	82.00	2.97	1.00	1.80	2.75	8.45	1.03
L. tulipifera	Oct.	82.00	2.97	1.00	1.80	2.75	8.18	1.30
V. rotundifolia	Oct.	82.00	2.97	1.00	1.80	2.75	8.68	0.79
fruits and vegetables								
apple		85.30	0.34	0.58	11.43	2.02	0.46	0.32
banana		73.90	1.15	0.18	20.03	1.82	0.56	0.83
Brazil nut		5.62	13.59	66.80	3.64	6.70		3.65
broccoli		89.70	3.30	0.20	2.51	3.00	0.33	1.10
cantaloupe[b]		85.40	0.90	0.10	12.40	0.73	0.08	0.40
carrot		88.20	0.98	0.20	4.80	3.63	0.26	0.86
cauliflower		91.60	2.46	0.28	2.34	2.92	0.22	0.82
celery		92.90	1.20	0.20	2.18	2.55		1.10
chick pea[c]		63.70	6.00	2.00	20.00	7.00		1.30
cucumber		96.80	0.60	0.20	1.81	0.54	0.26	0.60
French beans		90.30	2.39	0.24	5.09	1.89	0.20	0.72
grape[d]		81.10	0.68	0.28	15.24	1.50	0.35	0.48
kale[e]		86.30	4.30	0.90	2.54	4.20	0.44	1.70
kiwi fruit		83.80	1.00	0.63	9.12	2.12	1.49	0.72
mango		82.00	0.60	0.45	12.45	1.70	0.34	0.50
mustard, green[f]		86.30	4.30	0.90	2.54	4.20	0.44	1.70
onion		87.60	1.25	0.25	4.91	1.81	0.21	0.59
orange		85.70	1.00	0.20	8.25	1.60	1.13	0.48
pea, pod and seed, green[g]		75.22	6.55	0.48	12.30	4.25	0.28	0.92
pear		84.30	0.47	0.29	12.37	3.27	0.31	0.33
plum		83.70	0.60	0.17	10.16	1.58	1.25	0.49
red cabbage		91.80	1.50	0.18	3.54	2.50		0.67
sweetcorn[h]		74.70	3.28	1.23	15.72	4.22	0.05	0.80

species	water	protein	fat	carbo-hydrates	fibres	organic rest[a]	minerals / ash
sweet potato	69.20	1.63	0.60	24.08	3.14		1.12
watermelon	90.29	0.60	0.20	8.29	0.22		0.40
white cabbage	90.49	1.37	0.20	4.16	2.96	0.23	0.59
chow							
chow[i]	8.83	23.00	5.00	33.06	23.7		6.41

[a] The organic rest of fruits and vegetables (Souci *et al.*, 1994) consists of organic acids.

[b] The cantaloup(e) and honeydew melon, both available as such on the U.S. market, were both calculated as muskmelon (Souci *et al.*, 1994), the more as both sorts could be interpreted as vernacular names of different languages of *Cucumis melo* L. To indicate that two melon types were used as feed, cantaloupe was recorded as cantaloupe and honeydew melon as muskmelon.

[c] Canned chick peas (in water, salt and EDTA) were fed to the animals. The nutritional values of protein, carbohydrate, fat and fibre (in gram) are listed according to the declarations on the tag. The value for minerals is taken from Souci *et al.* (1994) of "chick pea, seed, green". The water content was calculated by difference method: 100 - (protein + fat + available carbohydrates + minerals + total dietary fibre).

[d] Grape: violet and red sorts were fed.

[e] Kale: frizzy and smooth sorts were fed.

[f] Due to lacking information on nutritive values, mustard green is calculated as kale.

[g] Two varieties of peas were fed.

[h] *Zea mays* L.

[i] The "Mazuri leaf-eater primate diet", No. 5672, Purina Mills, Inc., St. Louis, MO, quality controlled by PMI Feeds, Inc., was used as primate chow. The analyses values on the tag of the bag were as follows: Crude protein not more than 23.0 %; crude fat not more than 5.0 %; crude fibre 11.2 (NDF = neutral detergent fibre 23.7 %; ADF = acid detergent fibre 15.0 %); ash not more than 8.0 %; added minerals not more than 3.0 %.
For the total dietary fibre value, the NDF value was used. Crude water and ash were determined according to the "Weender methods of analyses" at the University of Veterinary Medicine, Hanover. The ash value is taken as value for minerals. The value for carbohydrates is estimated by difference method:
100 – (water + protein + fat + NDF + ash).

Contents of water and fat are set constant for fresh leaves, which limits interpretation of these values. However, the content of fat for the leaves is set constant at a maximum value to make sure that leaves are not underestimated so their maximum impact for the diet may be accurately assessed. Exceptionally high values in fat were recorded for Brazil nuts, with chow showing much reduced levels of fats. The inclusion of nuts within the lemur diets was sufficient to change the entire nutrient composition of the study groups (see Fig. C.1). Nevertheless, chow was rich in fat due to added soybean oil, which is declared on the nutritional tag. As will be seen in Fig. C.1, chow influences the amount of fat ingested by the sifakas. Other fruits and vegetables are generally low in fat.

The highest levels of "available" carbohydrates, that is non-structural polysaccharides and soluble saccharides, were found in chow, followed by sweet potato, chick pea and banana. The value of carbohydrates calculated for chow is higher than other values for non-structural carbohydrates aside the fruits & vegetables. Although leaves are generally low in soluble saccharides and starch, it is important to note that due to different analytical methods, the fibre fraction and the organic rest of the leaves also need to be taken into account as fractions may overlap.

Nevertheless, fruits are generally high in readily digestible carbohydrates, especially in simple saccharides. Most vegetables range in levels evidenced for the leaves, but depending on the plant part used, differences may occur. Starch was found to be highest in sweet potato, followed by sweetcorn and peas. Detailed values for chow were lacking. However, due to ingredients given on the tag such as ground yellow corn, corn gluten meal, ground oats and alfalfa meal, a rather high content of starch can be expected. Moreover, it is notable that *A. julibrissin* and *R. copallina* leaves of both seasons contain more starch than analysed for vegetables (Souci *et al.*, 1994).

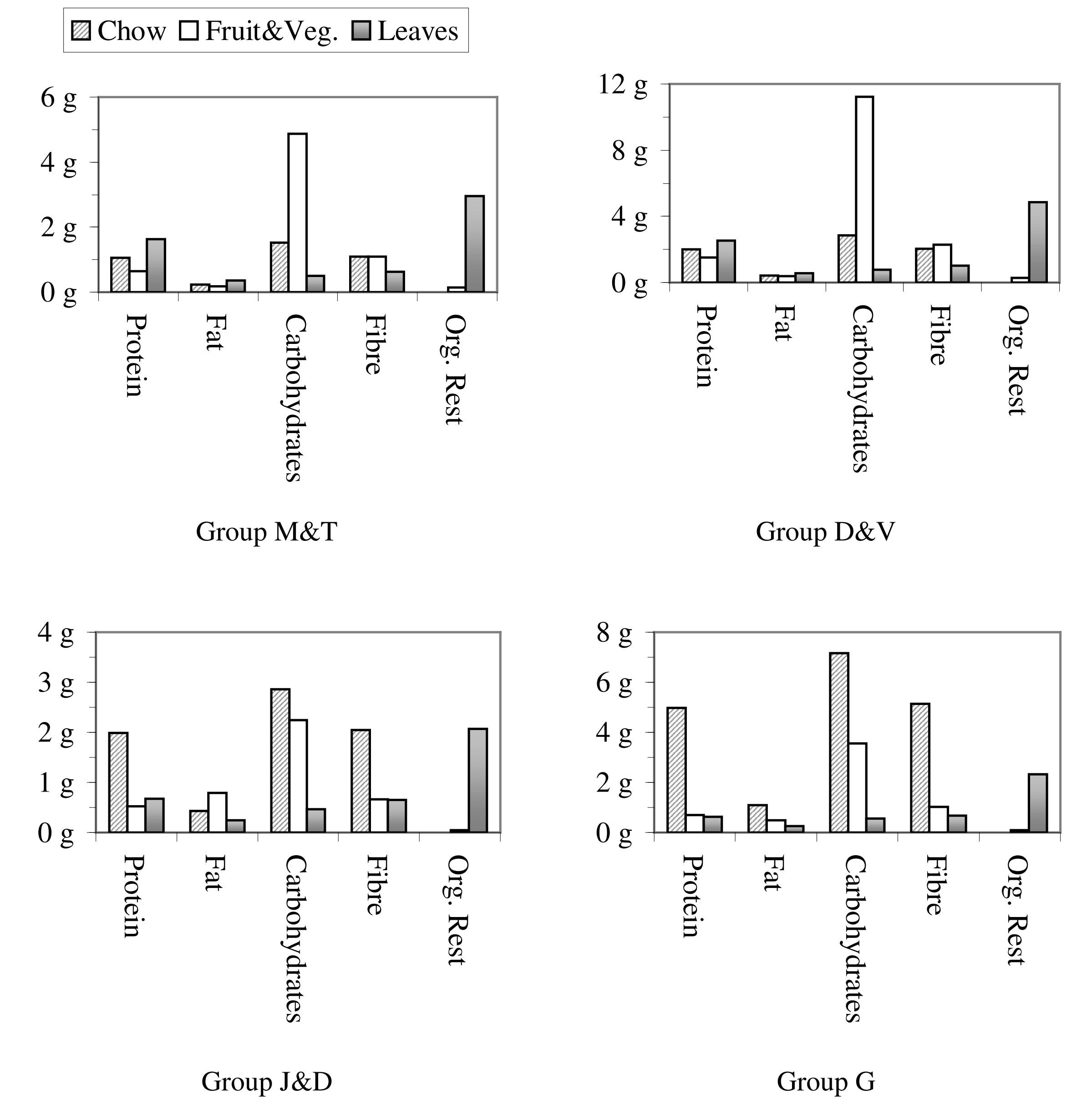

Fig. C.1: Weights (g / kg bodyweight and day) of the primary food constituents gained from the various food sources (based on fresh food weights) by the four groups of Coquerel's sifakas.

Apart from chow, chick peas and Brazil nuts were rich in fibres, followed by peas, sweetcorn and leafy greens such as kale. Primate chow is a specially designed high fibre food which is able to compete with leaves in this respect (crude fibre: 11.2 %, NDF: 23.7 %). Fibre values of the leaves are generally high and normally exceeding those of fruits and vegetables. However, a detailed evaluation of the leaves cannot be performed at that point due to the large organic rest with unknown composition.

Contents of “crude ash” determined for the leaves range within the upper level of contents for minerals determined for fruits and vegetables (Tab. C.3), indicating that leaves are certainly an important source of minerals.

As the study groups differed in number of animals and their respective bodyweights, food intake as well as ingestion of primary compounds was referred to kg bodyweight as the comparable entity.

In the following, the nutritional contribution of different types of food offered and their importance for the diet are scrutinised (Fig. C.1 and Fig. C.2). The composition of food constituents of individual food sources is principally reflected by the dietary composition of the sifakas. However, as the sifakas chose food sources to their liking, they have been able to manage an appropriate food composition regarding portions of main organic food constituents, such as carbohydrates, protein, fat, and fibres. Selected portions of leaves, fruits, vegetables and chow as the main food categories are ultimately responsible for the composition of primary compounds and fibre contents in their diet (Fig. C.1).

While Fig. C.1 shows the amounts ingested of primary food constituents including fibres and the organic rest gained from the main food categories (chow, fruits & vegetables and leaves), Fig. C.2 gives the percentages of the primary food constituents within the diet. With respect to food sources utilised, striking differences become visible among the four study groups observed.

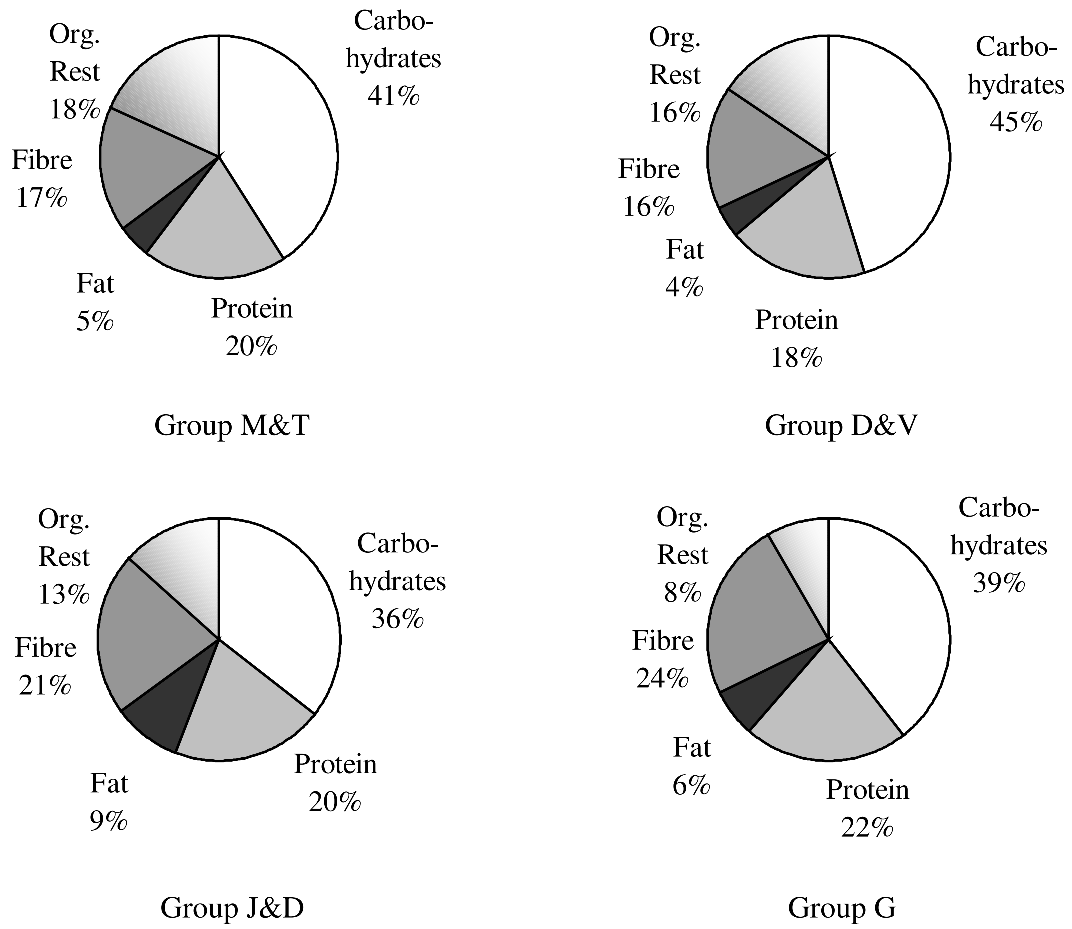

Fig. C.2: Nutritional composition in g/kg bodyweight (%) and day within each group’s diet referred to fresh weight of food.

During the spring season, the majority of protein was gained from leaves. Non-structural carbohydrates were mainly derived from fruits and vegetables. If the dietary fibre without the fraction of the organic rest is considered, chow, fruits and vegetables contain the most fibre. However, as previously mentioned, most water-soluble fibre types are contained in the organic rest of the leaves. Noting the fibre fraction within the organic rest, leaves are clearly the major source of fibre in the spring.

On the contrary, the diet in autumn was dominated by chow as the main source of nutrients. This is not surprising, if Fig. C.1 is compared to Fig. B.3 (p. 40) and Fig. B.5 (p. 42). Both groups in the autumn ingested rather large amounts of chow, a dry and nutrient rich commercial food source. While only a small portion of chow was given to and ingested by the groups in spring, large portions of chow were offered in the autumn and nearly half the weight ingested of fruits and vegetables was allotted to chow (Fig. B.3, p. 40). Due to its high nutrient density, chow competes with or even exceeds other food resources in nutrient supply. Hence, the diet in the autumn reflects the nutritional composition of chow. In Gordian's diet, chow clearly constituted the main source of fibre. However, as explained in chapter B, Gordian received few leaf species within his cage and in low amounts. As previously indicated, supply and demand constitute the major reason for the different portions consumed of the respective quantities of fruits & vegetables and chow during the spring and autumn months.

Levels of fat deserve a separate explanation. While most fat is gained from chow by the study groups in spring, the rather high fat levels in the diet of J&D and G originate from Brazil nuts, which were offered thrice to J&D and once to G. Moreover, chow contributes enormously to the supply of fat in autumn.

Despite different ages of the study animals and despite two different seasons, a review on food components reveals a clear dependence on age (Tab. C.4).

Tab. C.4: Averaged food composition[a] in g / kg bodyweight and day: groups of adult animals are placed above the broken line, juvenile and subadult animals are grouped below.

group	water	protein	fat	carbo-hydrates	fibres	org. rest	minerals	total[b]
M&T	70.9	3.3	0.8	6.9	2.8	3.1	0.9	17.8
J&D	37.1	3.2	1.5	5.6	3.4	2.1	1.0	16.7
D&V	135.0	6.0	1.4	14.9	5.3	5.1	1.7	34.5
G	51.4	6.3	1.8	11.3	6.8	2.4	1.9	30.5

[a] Weights are referred to fresh food based on Tab. C.3.

[b] Total ingested except for the content of water, which represents dry matter.
Total = protein + fat + carbohydrates + fibres + org. rest + minerals

Both groups with juvenile and subadult animals consumed nearly double the amount of total dry matter ("total", Tab. C.4) than adult animals, which reflects the higher nutrient requirements of growing animals.

In detail, young animals consumed nearly double the amount of proteins than adult animals. The same tendency nearly holds true for the fraction of available carbohydrates as major source of energy. If either the fractions of carbohydrates and fibres are added respectively for each study group, or if the respective fractions of carbohydrates, fibres and the organic rest are added, all calculations reveal that double the amount was ingested by groups with young, developing animals compared to groups of adults. Hence, soluble and structural carbohydrates are obviously essential sources of energy. In contrast, the amount of fat ingested does not reveal that clear dependence on the animal's age. This finding corroborates the suspicion that the amount of fat is more likely to be caused by food quality than by necessity to eat such amounts. The problem of ingesting "hidden" fats or more fat than needed for bodily maintenance is a well known cause of obesity in developed countries.

The amount of minerals ingested is likely to be a consequence of the quantity of food intake. Only a weak dependence on the animal's age becomes visible.

Water gained from food (Tab. C.4) is significantly higher for groups M&T and D&V observed in spring than for the study groups in autumn. Group D&V obtained the highest amount of water from the food as they ingested the largest portion of fruits and vegetables of all four study groups (Fig. B.3, p. 40; Tab. B.9, p. 43). As already indicated in chapter B, the low water supply for the groups in the autumn is obviously caused by the diet rich in primate chow, a sort of "dry food", which hardly contains any water. However, in contrast to spring, animals observed in autumn drank water, presumably to compensate for the low water content of the diet eaten. So, water intake from the food (Tab. C.4) is more likely to reflect the water content of the different food composition than actual needs.

Hence, in consideration of the varying food categories used, it is vital to determine whether the percentages (weight %) of the nutrients and dietary fibres may vary or are held constant in the diet, irrespective of food sources chosen and irrespective of the animals' age (Fig. C.2).

According to Fig. C.2 it is striking how constant percentages are among the study groups especially during the same season. Irrespective of the group's age, about 20 % (weight) were allotted to protein. While the percentage of fat averaged 4-5 % for the groups in spring, fat level raised to about 8 % for the groups in autumn, most likely due to the larger portion of chow and Brazil nuts ingested by the latter groups.

At first sight, carbohydrate and fibre levels resemble each other more for the groups of the same season than of different seasons. However, if the large organic rest is taken into account, differences between groups disappear considerably. The organic rest bears most imponderability as to chemical composition and its digestibility by the animals. It resembles on the one hand the fraction of dietary fibre and structural carbohydrates, and on the other hand the fraction of non-structural carbohydrates.

If the percentages of the fibre fraction and the respective organic rest are added, on average 34 % are consumed of that "enlarged" fibre fraction by all groups. Again, group J&D ingested the largest portion of leaves and a substantial amount of chow (Fig. B.3, p. 40), which contributes primarily to the quantity of dietary fibres and the organic rest fraction.

If the fractions of carbohydrates and fibres are added, on average 60 % are consumed by each of the four groups, while about 74 % are consumed if all three fractions, carbohydrates, fibres and organic rest, are added, respectively.

Hence, apart from the larger amount of fat ingested by the groups in autumn compared to spring (Fig. C.1 and Fig. C.2), nearly equal percentages are ingested of the other major organic compounds, that is protein, available carbohydrates and diverse types of fibre (Fig. C.2). This clearly emphasises the importance of these food constituents within the diet and underlines the constancy of food composition. Despite the large diversity of food specimens, nearly constant proportions of primary food compounds (including the diverse fibre fractions) could be examined for the diets of the different study groups, irrespective of the animal's age and season of the year. The major fraction of food, that is two thirds, are derived from non-structural carbohydrates and different types of fibres, which in turn consist mainly of polysaccharides. These findings are in accordance with the percentages recommended for the human's diet. An adult healthy human should consume at least 60 % (weight) of the diet as carbohydrates, best as starch, and 30-50 g fibres per day, that is (maximal) about 10 %. The remaining portion should be partitioned among proteins and fats.

3.2 Energy values of the food

In the following evaluation, energy requirements of the Coquerel's sifakas should be assessed with special emphasis on the contribution of the leaves to the diet. Although exact factors on the physiological needs of sifakas are still lacking, it was decided to use energy factors according to Souci *et al.* (1994), which are based on human's digestibility (C.I.2.3) and bear the advantage of being directly comparable to the recommended diet of humans. These factors calculating metabolisable energy are already used differently in animal nutrition (Conklin and Wrangham, 1994).

Hence, two different models are instigated to approach the energy supply of the food chosen by the sifakas. One approach is to evaluate the sifakas' diet like human's diet based on the capacities of the human's digestive tract, yielding minimum energy gain from the food selected. The other assessment takes into account the capability of midgut-fermenting monogastric mammals that use fibres as sources of energy, yielding maximum energy values from the diet chosen (Tab. C.5, Fig. C.3).

The maximum values account first for the capability of the large microbial flora to degrade dietary fibres, which contribute to the nutrient and energy supply by producing short-chain fatty acids. Hence, to take into account the fractions of dietary fibres and the organic rest as sources of nutrients and energy, both fractions are multiplied by the same factor as the fraction of available carbohydrates (17 kJ / g), which is in accordance with respective factors given by Meyer *et al.* (1993) for crude fibres and the nitrogen free extract (Tab. C.2). Other energy values determined by the bomb calorimeter (Tab. C.2) are not used in this work, as these values do not reflect the metabolisable energy. So, seen from the standpoint of energy supply, reality will be found between both limits, the minimum and maximum.

Tab. C.5: Energy contribution (%) of the major organic food constituents to nutrition for the four study groups. Maximum energy supply is calculated.

groups	protein	fat	carbo-hydrates	dietary fibre	org. rest
M&T	18 %	9 %	41 %	15 %	17 %
J&D	17 %	17 %	36 %	17 %	11 %
D&V	17 %	9 %	45 %	15 %	14 %
G	19 %	12 %	41 %	20 %	7 %

When comparing the percentages of weight (Fig. C.2) and proportions of energy percentages between individual food constituents, a consistent trend can be seen. As already indicated for the relation of weight percentages, no dependence on age can be discerned.

The diet of the four study groups consisted of approximately 18 % (energy) protein, which is roughly the same level as evidenced for the percentages of weights.

Energy contribution of fat to the diet is equal for groups in the spring, irrespective of their age, while it is increasing for the groups in the autumn. While the diet of Gordian contained 9 % (energy) fat, the largest amount is evidenced for the adult group J&D. As already indicated, this is more likely to be due to food quality ingested than to age dependent needs or due to seasonal reasons.

On average, 41 % of energy were contributed by the portion of non-structural carbohydrates to the diet. The lowest percentage of available carbohydrates in the diet is evidenced for group J&D, which is compensated by the largest percentage of fat. This is a common phenomenon within human's diet: that a large portion of fat replaces partly the percentage of carbohydrates while the portion of protein remains basically untouched. However, if the percentages of carbohydrates and fibres are added, these two fractions yield a total of 50-60 % (energy) of

the diet. If the organic rest is included additionally, the sum of these respective three fractions yield a total of 60-70 % energy. In a human's diet, about 60 % (energy) carbohydrates are recommended, with the best complex carbohydrates in the form of starches.
Fig. C.3 shows the energy contribution to the diet by the main food sources, chow, fruits and vegetables, and leaves. Especially for the groups in the autumn, chow contributed most to the energy supply of the diet. Again, due to its high nutrient density, chow supplies most energy in the autumn and a considerable amount in the spring to the respective group's energy budget despite its relative low intake. Although food intake per bodyweight differed largely between groups and despite varying quantities of different food sources ingested (Fig. B.5, p.42), it is amazing how animals apparently managed in an age dependent manner to achieve equal levels of energy gain (Fig. C.3).

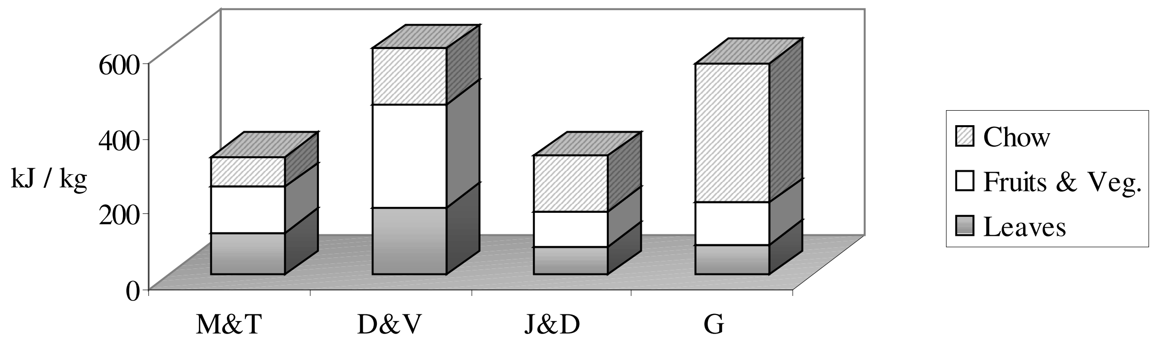

Fig. C.3: Maximum energy intake, in kJ / kg bodyweight and day, respectively for the four study groups.
Factors to convert weights of food (g) into their corresponding energy values (kJ) are presented in Tab. C.2.

With a total of (262-) 312 and (259-) 316 kJ / kg bodyweight were consumed by the adult groups M&T and J&D, respectively, energy intake amounted to (509-) 600 and (449-) 561 kJ / kg bodyweight respectively for the groups D&V and Gordian consisting of juvenile and subadult sifakas (calculated minimum and maximum energy values are given). Hence, nearly double the energy intake is requested by juvenile and subadult animals compared to adult groups. Supporting this finding is the fact that children of corresponding age require more than twice the energy than adult humans.

Obviously, in this model the portion of leaves contributes lowest to the energy supply of the total diet, although maximum energy values have been calculated in that model.

In a last attempt to evaluate the importance of the leaves in the diet with respect to energy supply, minimum and maximum energy values are calculated and their respective percentages are opposed to corresponding weight percentages of food intake (Tab. C.6).

Tab. C.6: Review on the portions of leaves (weight %) and their corresponding minimum and maximum energy supply to the diet, calculated respectively for the four study groups.

groups	weight	energy
M&T	38 %	23 - 35 %
D&V	33 %	18 - 30 %
J&D	45 %	13 - 24 %
G	31 %	7 - 14 %

Irrespective of the animal's age and season, at least 30 % by weight are allotted to the portion of leaves (Tab. C.6). Although their relative energy contribution to the diet varies considerably between minimum and maximum calculations, the overall energy contribution to the diet remains relatively low. Calculating maximum values, at best one third of the overall energy is delivered by the leaves for groups in spring. The far lower energy contribution of the leaves to the diet of groups in the autumn is due to the different food composition containing more food sources with rather high nutrient densities like chow and nuts. Despite these differences leaves and especially two species, *R. copallina* and *A. julibrissin*, remain highly selected food items.

4 Discussion

There is no doubt that nutrition has to meet the needs for at least minimum amounts of certain nutrients, vitamins and minerals for a given animal species (Milton, 1979; 1980; Langer, 1988; Stevens, 1988). On the other hand, the digestive system of the animal host and its microbial flora determine both the food quality and quantity required for the animal's well-being (Langer, 1988; Stevens, 1988; Hofmann, 1983; 1991; Onderdonk, 1998). Digestion is a species-specific process, which depends on an animal's age, state of health and adaptations of the gut microflora to the food (Meyer *et al.*, 1993).

At present, different primary food constituents are discussed that influence food choice. Generally, in a natural diet, leaves are a valuable source of protein, dietary fibres and minerals. For non-structural carbohydrates like simple saccharides and starch, fruits and vegetables are an appropriate source. In the wild, both fruits and nectar rich flowers provide animals with readily absorbable nutrients and energy (Richard, 1978a, b; Milton, 1979; 1980; Powzyk, 1997). For free-ranging folivores (Milton, 1979; 1980; Glander, 1982) leaf contents (i.e. protein and fibres) tend to be crucial to leaf choice. There is some evidence that a common feature in the feeding pattern of many herbivores is a preference for immature over mature leaves, which normally contain higher contents of protein and lower levels of dietary fibres than mature leaves (Milton, 1979; Glander, 1982; Waterman, 1984).

In this study, *A. julibrissin* leaves of all seasons analysed were high in protein levels, while *R. copallina* leaves range in more medium levels (App. II.1), which is supported by recent studies (Campbell *et al.*, 1999; 2001). Compared to analyses performed on various leaf species, which are food plants of different free-ranging prosimian species in Madagascar (Ganzhorn, 1988; Powzyk, 1997) and of howler monkeys (Milton, 1979; 1980), it can be stated that the protein contents of *A. julibrissin* leaves can be classified as extremely high. Surprisingly, values found for protein intake by adult groups of *P. v. coquereli* (approximately 3 g / kg bodyweight per day) equal values found for *P. v. coronatus* at the Zoological Park of Paris (Lopes Santini, 1992), and those calculated for howler monkeys (Milton, 1979; 1980). The National Research Council (1978) recommends 3.5-4.5 g crude protein per bodyweight for adult Old World primates. The lower level is in accordance with amounts eaten by adult sifakas in this study. In contrast, for adult humans and children[1], 0.8-1.2 g protein per kg bodyweight and day are recommended (Biesalski *et al.*, 1995; DGE 2005). In contrast, the total sifakas' diet is pronounced in absolute amounts of proteins, albeit the respective energy contribution to the diet ranges within recommendations for humans (5-20 %) (Biesalski *et al.*, 1995).

Like a human's diet, the portion of fat is difficult in its need to be held constant while yielding highest energy gain of all nutrients and being highly digestible as evidenced for humans and *P. v. coronatus* (Lopes Santini, 1992; Biesalski *et al.*, 1995). Compared to *P. v. coronatus*

[1] children older than one year

(Lopes Santini, 1992), fat intake resembles more the adult group M&T than J&D. Differences in fat consumption between groups of spring and autumn are more likely to be due to different food specimens used than due to different needs and requirements. However, compared to human's diet with fat contributing 30-35 energy % to the total diet (Biesalski *et al.*, 1995), the sifaka's diet is extremely low in fat.

It is striking that the largest portion of nutrients is constituted by complex carbohydrates like starch, and soluble saccharides. Comparable to *P. v. coronatus* (Lopes Santini, 1992) as well as to a human's diet, carbohydrates constitute the major source of energy in this study. For humans and presumably for primates in general, carbohydrates are the best source of energy, which should not be replaced by either protein or fat and are directly available to the organism. Lopes Santini (1992) found an apparent digestibility of 100 % for non-structural carbohydrates in *P. v. coronatus*. Normally, the brain gains nearly all its energy from glucose. Another theory indicates that satiety is only reached if enough carbohydrates are ingested. However, the intake of proteins and available carbohydrates show the clearest dependence on the animal's age (Tab. C.4) and resemble in portions the human's diet.

Fibre values are somewhat sophisticated to be evaluated. Generally, dietary fibres are comprised predominantly of cell wall structures, namely a polysaccharide-type such as cellulose, hemicellulose, and pectins, but also products like gum and plant mucilage. The latter differ in their water solubility and gelling properties, and to lesser extent lignin as water-insoluble phenolic polymer. In this context, it should be noted that cellulose is the major constituent of cell walls in higher plants, providing 20-40 % of the dry matter (Stevens, 1988).

The different types of fibres occur in varying amounts in the leaves, depending on season and the leaf's age (Milton, 1979; 1980; Glander, 1982; Hofmann, 1983; Ganzhorn, 1988; Powzyk, 1997), and reveal important influences on the gut functioning, transit time and the microbial ecosystem of the animal host (Wolin, 1981; Plaut, 1984; Marcus and Heaton, 1986; Savage, 1986; Stevens, 1988; Carman *et al.*, 1993; Onderdonk, 1998). Different fibre types influence the digestibility of other nutrients, water and electrolyte absorption and the binding of endogenous compounds like bile acids and cholesterol (Wolin, 1981; Ehle *et al.*, 1982; Plaut, 1984; Marcus and Heaton, 1986; Savage, 1986; Langer, 1988; Milton and Demment, 1988; Stevens, 1988; Meyer *et al.*, 1993; Mendeloff, 1984; Carman *et al.*, 1993; Zentek, 1996).

Regardless of the carbohydrate substrate and the microbial flora of forestomach- and midgut-fermenting mammals, they all produce predominantly short-chain fatty acids and gases. In turn, short-chain fatty acids produced by the microbial flora have an important influence on e.g. absorptive processes, pH of the interior milieu, gut motility, bacterial growth and hence the microbial ecosystem and state of health of the animal host (Wolin, 1981; Ehle *et al.*, 1982; Mendeloff, 1984; Plaut, 1984; Milton and Demment, 1988; Stevens, 1988; Zentek, 1996). The extent and ratio between acetic, propionic and butyric acid, the rate of fermentation, the pH in the fermentation chamber that in turn has impact on the microbial colonies in number and composition, this all is largely influenced by food quality and quantity with respect to the composition of structural and non-structural carbohydrates. Different proportions between simple saccharides, starches, and structural carbohydrates in the diet have a massive impact on fermentative and absorptive processes of electrolytes and in many respects, the nutrients themselves (Stevens, 1988; Meyer *et al.*, 1993). In humans, short-chain fatty acids play an integral role in maintenance of colonic integrity and metabolism, and are proved to be used as fuel for colonic epithelial cells and to have trophic effects on the epithelium (Cook and Sellin, 1998).

The digestibility of the different fibre types cellulose, hemicellulose, pectins and lignin varies considerably between animal species (Lopes Santini, 1992; Campbell *et al.*, 2002; Campbell *et al.*, 2004b), which largely depends on the equipment of the digestive tract and associated organs. Normally, digestibility decreases from hemicellulose to cellulose and is lowest for lignin, while pectins and gums are rapidly and fully fermented, yielding different amounts of short-chain fatty acids as a source of energy (Milton, 1979; Hoogkamp-Korstanje *et al.*, 1979; Ehle *et al.*, 1982; Stevens, 1988; Van Soest, 1994; Lopes Santini, 1992; Campbell *et al.*, 2002; 2004b). Lopes Santini (1992) found that captive *P. v. coronatus* had an apparent digestibility of about 74 % for hemicellulose, 65 % for cellulose and 62 % for lignin. Depending on experimental conditions *P. v. coquereli* showed an apparent digestibility of about 53 % for insoluble fibres and 65 % for soluble fibres, while the digestibility of the acid detergent and neutral detergent fibre fractions varied greatly (Campbell *et al.*, 1999; 2004b). For *P. tattersalli* slightly deviant values had been found (Campbell *et al.*, 1999).

In this context the detection of the plant mucilage in *A. julibrissin* (chapter C.V) becomes critical. Despite its pharmacological properties, which will be discussed in chapter C.VI, it can be presumed that this pectin-like mucilage will be largely degraded in the fermentation chambers of the midgut by the microbial flora yielding short-chain fatty acids. As in this study the plant mucilage is contained in the organic rest of the leaves, the fraction of fibres plus the organic rest are taken into account for calculations on maximal energy gain from the leaves. The content of dietary fibres thus calculated[1] reaches levels that are commonly found in other leaf species (Milton, 1979; Ganzhorn, 1988; Powzyk, 1997).

Furthermore, it becomes visible, that even lignin is largely degraded by the microbial flora of the different *Propithecus* (sub-)species, which fits to the observation that *P. v. coquereli* of this study ingested dead needles of *Pinus taeda* and sometimes gnawed at bark or wood. Similar observations are reported from free-ranging *P. v. coquereli* and *P. v. verreauxi* in Madagascar (Richard, 1977). In this context, the role of seed ingestion by *P. d. edwardsi* (Hemingway, 1996; 1998) and e.g. the cotton-top tamarins (Heymann, 1992; Garber and Kitron, 1997), presumably deserves more appreciation for their effects on the gastrointestinal tract.

However, focusing on primary compounds including fibres as source of energy, there are more reasons to plead for leaf ingestion in general, than for the leaf choice selected by the sifakas. While *R. copallina* leaves were ingested in spring and autumn alike, animals discriminated between spring and autumn leaves of *A. julibrissin*. In the cooler months of autumn, far less *A. julibrissin* leaves were eaten compared to spring, and if at all, younger autumn leaves were preferred (see chapter B). This is surprising, as the older autumn leaves contain more protein and less fibre levels than the younger autumn leaves. On the contrary, *R. copallina* leaves, which were highly eaten in both seasons studied, contain far less protein and partly more fibres than accepted by the animals in the case of autumnal *A. julibrissin* leaves (App. II.1). From the nutritional point of view, neither protein, nor fibre levels of autumnal *A. julibrissin* leaves explain the decrease in selecting this leaf species by the animals.

Another aspect for the general use of leaves in the diet is their leaf specific composition. Leaves are obviously an appropriate source of food to reach an equilibrium between individual nutrients, as they are low in fat, low in simple saccharides and rich in polysaccharides. These findings are corroborated by results of Lopes Santini (1992) on the food composition of captive *P. v. coronatus*. For captive low-land gorillas, health improved much and regurgitation and coprophagy vanished by supplementing the diet with large amounts of fibre (Rümpler, 1990; Müller and Schildger, 1992). Increased portions of fibre and a reduction in fat was effective to treat obesity in captive orang-utans, gorillas and to prevent subsequent metabolic diseases similar to those described for overweight humans (Markham, 1990; Müller and Schildger, 1992). Female sifaka tend toward obesity when

[1] Compared to gross energies gained from the short-chain fatty acids yielded by microbial degradation (Tab. C.2), the factor used in this work is at the lower level and in agreement with the fraction of carbohydrates and the nitrogen free extract.

offered an excess of high-quality foods and could benefit from ingesting leaves as a weight control measure (Glander *et al.*, 1993; Campbell *et al.*, 2001).

The role of certain minerals and vitamins is not further regarded in this study. However, minerals and vitamins are essential for enzymatic processes and deficiency as well as an overload may be detrimental. Hemosiderosis in lemurs and calcification in *P. d. diadema* are examples of dietary overdoses with lethal ending (Spelman *et al.*, 1989, Powzyk, pers. comm.).

Another important aspect of the sifakas' diet is obviously the intake of water. Drinking behaviour seems to build an important clue to understanding feeding behaviour and hence the physiology of sifakas themselves. In this study, animals were never seen drinking in spring and were only sometimes observed drinking in the autumn. Differences in the drinking behaviour between spring and autumn might be due to a higher water content of immature versus mature leaves, a larger supply of juicy fruits, and presumably the lower intake of dry primate chow in spring compared to autumn.

However, the general feature of a healthy animal with no or only few drinking could be an indication to the capability of nitrogen recycling. In forestomach- and midgut-fermenting mammals, the indigenous microbial flora is capable of converting protein and nonprotein nitrogenous compounds of endogenous and exogenous origin into microbial protein, that is subsequently digested and absorbed by the animal host. One major source of nonprotein nitrogen is urea, which enters the gastrointestinal tract by direct diffusion from the blood. This allows for both the synthesis of high-quality protein from low-quality diets and the conservation of nitrogen. In this context it is interesting to note that captive *P. v. coquereli* and captive *P. tattersalli*, showed a remarkable increase in blood urea levels when consuming an experimental diet rich in protein (Campbell *et al.*, 1999). The recycling of urea in turn allows for the conservation of water that would otherwise be required for urinary excretion (Bauchop, 1978; Parra, 1978; Wolin, 1981; Langer, 1988; Stevens, 1988). Furthermore, utilisation of nitrogen and fermentation of carbohydrates are interdependent, and restriction of water normally increases the rate at which urea is recycled, as evidenced for temperate, tropical, and desert ruminants (Stevens, 1988). The ability to survive without drinking water is also reported from other arboreal folivores like langurs (Bauchop, 1978), howler monkeys (Glander, 1982), *P. d. diadema* and *Indri indri* (Powzyk, 1997), *P. d. edwardsi* (Hemingway, 1998) and the koala[1] (Zoidis and Markowitz, 1992).

In order to assess the dietary role of the leaves in this study, these trends have to be taken into account, that under captive conditions there are less constraints to ingest leaves as a source of protein and/ or fibres than in the wild with seasons of fruit scarcity and seasonal fluctuations in food availability (Richard, 1978a, b). In many respects, commercial primate chow can be regarded as valuable substitute for a range of natural food sources (Tab. C.3). It is high in protein, fat, available carbohydrates, fibres and minerals compared to most other food items. In autumn, Brazil nuts could be used especially as source of fat. However, except for nuts, chow exhibits the highest nutrient density of all other food sources supplied. Hence, the larger consumption of chow by animals in autumn compensates for the lower food intake and obviously for the lower leaf intake per bodyweight (Fig. B.5, p. 42; Tab. B.9, p. 43).

In part, vegetables fed to the focal animals compete with or even exceed leaves in contents of protein, fat and fibres. Like in the wild, fruits are the main source of readily available carbohydrates, especially of simple saccharides (Fig. C.1). Hence, from this point of view, there is actually no need to select leaves in captivity. However, despite a superabundance of nutrients in captivity, leaves are an indispensable portion of their total food intake with a clear predilection for two leaf species.

[1] Koala is an aborigine name for "animal that does not drink."

Regarding nutrient composition and energy supply to the sifakas of this study, it is striking that despite different sources of food that supply different nutrient compositions in quality and quantity, animals were obviously capable to attain nearly constant levels of nutrients and energy gain per bodyweight with a clear dependence on the animal's age (compare Fig. B.5, p. 42 versus Fig. C.3; Tab. B.9, p. 43 versus Tab. C.4).

In this study an average of 314 kJ per kg bodyweight has been calculated for adult sifakas, while juvenile and subadult sifakas consumed nearly double the amount, when maximum energy gain from the leaves was assumed (Fig. C.3). Neglecting energy gain from the portion of fibres, an average of minimal 259 kJ per kg bodyweight has been calculated for adults. These findings for adult *P. v. coquereli* of this study agree with calculations on metabolisable and digestible energies for captive *P. v. coronatus* and *P. v. coquereli*, while for *P. tattersalli* even lower values have been found (Lopes Santini, 1992; Campbell *et al.*, 1999). For *P. v. coronatus* an average of 355 kJ (metabolisable energy) per bodyweight has been noted (Lopes Santini, 1992), 271 kJ (digestible energy) per bodyweight for *P. v. coquereli* and 225 kJ per bodyweight for *P. tattersalli* (Campbell *et al.*, 1999). In contrast, except for *P. tattersalli*, gross energy[1] intakes resembled those recommended for Old World primates by the National Research Council (1978) with up to 420 kJ per bodyweight (Lopes Santini, 1992; Campbell *et al.*, 1999).

Furthermore, for most (if not all) prosimian species (Müller, 1983; Richard and Nicoll, 1987; Richard and Dewar, 1991; Ross, 1992; Lopes Santini, 1992) as well as a range of arboreal folivores such as tree sloths (*Bradypus*, *Choloepus*) and marsupial tree kangaroos and koalas (Bauchop, 1978; McNab, 1978; Parra, 1978), a reduced metabolic rate associated with a reduced body temperature is in evidence. In these animals, body temperature is more susceptible to environmental fluctuations than what is found in homeothermic mammals. Presumably, prosimians exhibit an energy expenditure reduced in 10 to 60 %, which is associated with a lower metabolic rate (Lopes Santini, 1992).

With respect to the portion of leaves, in spring an average of 36 % leaves (by weight) were consumed by both groups, which delivered a maximum of 144 kJ per bodyweight and attributed 33 % metabolisable energy to the diet. In the autumn, groups consumed a mean of 38 % leaves by weight, gained a maximum of 77 kJ per bodyweight, which contributed with 19 % to the energy of the diet. In a study by Campbell *et al.* (2001) when animals were fed a diet enriched in chow, browse then contributed 30 % by weight to the diet yielding 27 % gross energy. Overall, the energy contribution of the portion of leaves to the diet remained rather low, irrespective of the animal's age and irrespective of other food sources available (Tab. C.6).

5 Conclusion

It can be concluded, that leaves were not ingested for reasons of mere nutrient and energy gain. Although it can be presumed that leaves are a valuable source of protein, minerals and fibres, there was a superabundance of other sources of food provided to supply nutrients and energy in this study. The idea that leaves are required to get a balanced food composition as to primary food constituents including fibres pleads for more overall ingestion of leaves, but did not support the type of leaf selection exhibited by the sifakas in this study. However, this work did reveal a clear predilection for two leaf species, *R. copallina* and *A. julibrissin*, with seasonal differences for the latter species. Hence, additional factors with impact on leaf choice will now be investigated: the role of secondary plant compounds.

[1] For calculations of energies see Tab. C.2.

C.II Evaluation of the food plants based on plant chemistry with emphasis on chemotaxonomical and pharmacological aspects: A literature survey

The investigation of the nutritional and energy values of foods consumed (chapter C.I) did not conclusively explain why animals selected certain leaf species as food sources. Therefore, the role of the secondary plant compounds including possible inherent pharmacological effects will now be surveyed prior to any analytical investigations. This survey will be categorised according to the groups of secondary plant compounds inherent to the different plant species and parts. Special emphasis will be placed on the two major food species, *Rhus copallina* and *Albizia julibrissin*. Relevant chemotaxonomical implications will also be presented.

Chemotaxonomy and its known chemical relationships between species, genera, families and orders is normally a useful means to establish and verify plant systematic. In this work, chemotaxonomy is used as a key to a comprehensive survey on secondary plant compounds occurring in each plant species and plant part which are found within the environments of the focal lemurs (App. II.2). I will also assess the possible impact of these plant compounds on the animals' food choice, which is mirrored by their feeding behaviour.

All information gathered in App. II.2 focuses on the plant species ingested, especially their leaves, but also takes into account those species, that were refused or remained untouched within the natural habitat enclosures. When information was scarce on the level of a definite species, the respective genus or family was regarded including general implications of the plant order, keeping in mind that natural products even characteristic on the familial level may not occur in an individual species or in the respective plant part chosen by the animals (App. II.2; Tab. B.7, p. 34). Hence, for a reliable evaluation of secondary compounds which might be actively sought out, merely tolerated, minimised, or avoided by the animals, a most comprehensive knowledge of each plant species and its plant parts accessible to the animals, will be indispensable in discerning the necessary requirements of food quality to maintain lemur health. It is presumed that only on the level of chemical groups of secondary plant compounds, tendencies to feed on plant species or the avoidance of plant species can be discussed.

Known pharmacological effects of the plant species and their compounds were included with special reference to possible benefits for the sifakas (App. II.2). Whenever available, the contents of chemical plant compounds were included. Furthermore, related plant species of a respective genus growing in Madagascar were also discussed.

Tannins

The ubiquitous group of tannins seems to play a vital role in food choice and overall diet of many lemur species (Spelman *et al.*, 1989). Free-ranging members of the indriid family preferred leaf and fruit galls as food items whenever available. In Madagascar, *P. d. diadema* sought out galls from species of the Anacardiaceae and Myrtaceae, while *Indri indri* ingested galls from members of the Euphorbiaceae and Lauraceae (Powzyk, 1997), which can be interpreted as an indication of (hydrolysable) tannin selection.

Rhus copallina leaves were the most preferred leaf species by all focal sifakas during both study seasons and are expected to contain large amounts of hydrolysable tannins (App. II.2). Hegnauer (2001; App. II.2) reported high yields from "three geographic races" of *R. copallina*, such as 27 %, 33 % and 37 % of gallotannins in their leaves (referred to dry weight), which obviously represents high-yield cultivars that were obtained during a commercial cultivation program initiated in the USA in 1965. This cultivation program was performed with *Rhus glabra* and *Rhus copallina* as the most common wild *Rhus* species to yield USP quality tannic acid (Doorenbos, 1976; Doorenbos and Box, 1976; App. II.2). Investigation of leaves, flowers, stems, barks, woods and seeds of eight North American *Rhus* species revealed high tannin contents in both the leaves and flowers with lower levels occurring in other plant parts (Hegnauer, 2001; App. II.2). The ingestion of leaves from these high-yield cultivars fits well with desired antidiarrhoeal effects and targets the main malady of captive indriids. In addition, flowers have also been reported to be ingested (DULC staff, pers. comm.).

Other preferred leaf species that contain a predominance of hydrolysable tannins (App. II.2), are the leaves of *Acer rubrum* (Aceraceae) and *Liquidambar styraciflua* (Hamamelidaceae).

From a chemotaxonomical point of view it is interesting to note that the Aceraceae are related to the Anacardiaceae, while the Anacardiaceae are similar in chemical composition to the Hamamelidaceae, with all families accumulating tannins (Hegnauer, 2001). Accordingly, leaves of *A. rubrum* (Aceraceae), *R. copallina* (Anacardiaceae) and *L. styraciflua* (Hamamelidaceae) were highly selected by the focal animals.

In the realm of traditional human medicine, the barks of *L. styraciflua*, *A. rubrum* and *Cercis canadensis*, have been recommended for gastrointestinal disorders such as dysentery and diarrhoea or were used as a tonic, for which high contents of condensed tannins were reported or may be postulated (App. II.2). However, these barks were not ingested by the focal lemurs. This fits with the observation that leaves and barks of oak trees (i.e. different species of the genus *Quercus*, Fagaceae) with their known contents of condensed tannins, were just tasted or remained untouched by the focal lemurs. Evidently, the bark and wood of these plant types were not selected based on their high content of condensed tannins.

The role of *Robinia pseudoacacia* leaves is somewhat contradictory and needs further investigation (compare own chemical analyses, chapter C. IV). For *R. pseudoacacia* leaves, the occurrence of condensed tannins has been documented (Kumar and Horigome, 1986; App. II.2). While *R. pseudoacacia* leaves were less preferred by the focal *P. v. coquereli* at Duke, leaves are reported to be preferred by captive *P. v. coronatus* at the Zoo of Paris (Lopes Santini, 1992). *R. pseudoacacia* leaves are known to provoke fever, oedema and obstipation, and in homeopathy, fresh barks and young twigs are indicated for gastric disorders, ulcers, obstipation and headache (App. II.2). However, *R. pseudoacacia* leaves were eaten by both *Propithecus* subspecies, albeit with different impetus.

Therefore, it can be concluded, that tannins play an important role in leaf choice with the focal *P. v. coquereli* and other lemur species. The focal *P. v. coquereli* show a clear discrimination between hydrolysable tannins (gallo- and ellagitannins) and condensed tannins (proanthocyanidins) yet there is a clear preference for the hydrolysable type, albeit condensed tannins may be tolerated to a limited extent. Nevertheless, the content of hydrolysable tannins may overrule simultaneously present condensed tannins.

Although both condensed tannins (e.g. Quercus cortex) and hydrolysable tannins have been used generally as an antidiarrhoeal in traditional human medicine, the German pharmaceutical market utilises the hydrolysable type of tannins predominately in the field of diarrhoeas (tannin albuminate; e.g. Tannalbin®), which is in agreement with the sifaka's preference for the hydrolysable tannin type.

Flavonoids

Various flavonoids have been isolated from the stem bark of *Albiz(z)ia julibrissin* (Tribus Ingeae, Mimosaceae) (Jung *et al.*, 2004a; App. II.2), albeit it were the leaves that were selected as one of the most preferred food items by the focal lemurs. Quercitrin and isoquercitrin have been identified within their flowers (Kang *et al.*, 2000; App. II.2). For the latter two compounds, sedative effects were shown which support the utilisation of flowers as a sedative drug in traditional oriental medicine (Kang *et al.*, 2000). The occurrence of hyperoside in *A. julibrissin* is mentioned without giving the exact plant part of origin (App. II.2). Quercitrin and afzelin have recently been reported to occur within the leaves, for which free radical scavenging activities have been found, respectively (Jang *et al.*, 2002; App. II.2).

The leaves of *Albizia lebbeck*, a Malagasy food plant of *P. tattersalli* (Meyers, 1993), are reported to contain quercetin- and kaempferol-tri-O-glycosides (El-Mousallamy, 1998). Although the exact plant organs ingested are not reported (Meyers, 1993), the knowledge and ingestion of flavonoids by free-ranging *Propithecus* species seem certain.

It is notable that flavonol glycosides, particularly when present with quercetin, kaempferol and to a lesser extent with myricetin as aglyca, and flavone glycosides which contain apigenin or luteolin aglyca, are widely distributed within plant kingdom and were described to occur in many leaf species selected and not selected by the sifakas (compare App. II.2). In most selected leaf species, flavonol glycosides especially with quercetin and kaempferol as aglycone moiety seem to be abundant, e.g. in the leaves of *A. julibrissin*, *Liquidambar styraciflua*, *Liriodendron tulipifera* and in the genus *Cercis* with *C. canadensis* as a selected leaf species (App. II.2), which was verified by subsequent chemical investigations in this study (chapter IV). Various flavone glycosides are expected to occur in the leaves of *R. pseudoacacia* (compare App. II.2 and chapter IV).

In this context it is notable to mention the chemotaxonomic proximity between the Caesalpiniaceae, Fabaceae (= Papilionaceae) and Mimosaceae of the order Fabales[1] (= Leguminosae), which provided important food plants for the focal lemurs: *C. canadensis* (Caesalpiniaceae), *R. pseudoacacia* (Fabaceae), and *A. julibrissin* (Mimosaceae). The proximity of the three families is emphasised by their alternative division into the three subfamilies Caesalpinioideae, Mimosoideae and Papilionoideae (Hegnauer, 2001).

In contrast, other compounds may overrule the present flavonoids: *Carpinus caroliniana* leaves were just tasted, albeit they are known to contain quercetin glycosides. The tree *Ailanthus altissima* was only passed while the leaves contain quercetin, kaempferol, apigenin and luteolin glycosides, and *Ulmus alata* leaves were refused and contain myricetin glycosides (App. II.2). Moreover, the order Urticales, containing the Moraceae (*Morus rubra*) and Ulmaceae (*Ulmus alata*), provides an example of an inconsistent feeding behaviour in regard to species acceptance and avoidance and in regard to individual feeding habits of the focal lemurs despite a strong chemotaxonomical relationship between the Moraceae and the Ulmaceae.

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[1] The order Fabales is characterised by its rich occurrence of tannins of various types, flavonoids, and the frequent occurrence of oleoresins, gums and plant mucilage. The large and distinct metabolism of flavonoids in its entity can be evaluated as characteristic of leguminous plants. The flavonol aglyca kaempferol and quercetin are widely occurring and more frequently than myricetin (Hegnauer, XIa, 1994).

While the leaves of *U. alata* had been spat out, *M. rubra* leaves were eaten differently by Marcella and Tiberius. Whereas Marcella ate the leaf (tip and / or blade), Tiberius ingested the stalk including the white latex. The leaves are reported to contain flavonoids, while nothing is reported about the latex so far. Hydrolysable tannins are largely missing in the Moraceae and hence are not expected to occur in the leaves. Tannins are not typical of this plant order yet if present, the condensed tannins would predominate (App. II.2). Simple phenolics and stilbenes occur in the wood of *M. rubra*, while the bark is reported to exhibit laxative and anthelminthic properties, yet neither part was ingested by the focal lemurs. The leaves of *U. alata* are known to contain myricetin glycosides, and condensed tannins may be expected. However, it cannot be ultimately reasoned as to why the leaves of *U. alata* were refused.

Therefore, it can be concluded that flavonoid glycosides are among the preferred chemical compounds, but may be overruled by other simultaneously occurring compounds decisive for leaf choice. In subsequent chemical investigations a rich occurrence of flavonol glycosides was found in the leaves of *A. julibrissin* and other selected leaf species (chapter IV). The role of flavonoids, their impact on leaf choice, and possible beneficial effects on the gastrointestinal tract will be further surveyed and discussed in chapter VI.

Stilbenes

Stilbenes are documented to occur in the genus *Vitis* (in leaves and wood) (App. II.2). While resveratrol and its derivatives were found to occur in the wood of different *Vitis* species, they were only synthesised within the leaves when stressed (after infection with different fungal strains and exposure to UV-light). The fungitoxic properties, especially of the oligomeric resveratrol derived compounds of the leaves have classified them as phytoalexines (Hegnauer, 2001). However, leaves and wood of *Vitis cinerea* and *V. rotundifolia* (Vitaceae) were tasted in the spring and consumed during the autumn (App. II.2). Hence, the role of stilbenes for food selection cannot be fully addressed, however these compounds may be of minor importance.

Simple phenolics (cinnamic acids, urushiols[1], naphthoquinones and coumarins)

The group of **hydroxycinnamic acids** is widely occurring within plant kingdom. Most compounds occur as esters, seldom as free acids (Rimpler, 1999). They are often associated with tannins and flavonoids and were ingested by the focal animals (App. II.2).

Phenolic compounds with irritating effects such as **urushiols** were obviously avoided by the focal lemurs. *Rhus radicans* (Anacardiaceae) covered the ground level of the natural habitat enclosure No.1 and remained untouched, presumably due to its latex that contains skin irritating phenolics (urushiols) which can cause *Rhus* dermatitis (App. II.2). A latex with irritating phenolics has been described for *Mangifera indica* (another Anacardiaceae) and may be the reason for the careful selection of plant tissue by the animals. In Madagascar, *M. indica* is known to be a food plant for *P. tattersalli* (Meyers, 1993), although the plant parts consumed were not specified.

The evaluation of plant species and plant parts containing **naphthoquinones** is somewhat sophisticated as simultaneously occurring chemical compounds such as tannins make interpretations more problematic. Furthermore, content information on certain plant parts is often lacking. Thus the evaluation of *Diospyros virginiana* leaves (Ebenaceae) is not unequivocal. Naphthoquinone derivatives exhibiting various pharmacological effects and tannins, predominantly of the condensed type, are widespread in *Diospyros* and occur in many plant organs, although the naphthoquinones were not explicitly reported in the leaves of *D.*

[1] 3-alkyl- and 3-alkenyl-brenzcatechins with C_{15} or C_{17} side-chains (Frohne and Jensen, 1992)

virginiana (Hegnauer, 2001; App. II.2). The leaves were tasted in spring and eaten in the autumn by the focal lemurs. This raises the question, whether the content of naphthoquinones fluctuated dramatically according to the season, or whether the study groups had distinct food preferences. Conversely, different *Diospyros* species are reported to be utilised as food plants in Madagascar by *P. tattersalli* (Meyers, 1993), by *P. verreauxi* in Morondava (leaves; Ganzhorn, pers. comm.) and by *P. v. verreauxi* (young leaves; Ranarivelo, pers. comm.; Richard, 1978a).

Another species, *Carya tometosa* (Juglandaceae), explicitly leading naphthoquinones in the leaves (Hegnauer, 2001; App. II.2), was merely tasted by the focal lemurs, corroborating the impression that high contents of naphthoquinones were avoided.

The bark of *D. virginiana* containing naphthoquinones (Hegnauer, 2001) is known to act as febrifuge (like cinchona) and as an antiseptic (Crellin and Philpott, 1990; App. II.2). But contrary to human's use, plant parts with anti-malaria and febrifuge effects were not explicitly selected by the focal animals, as was already shown for the bark of *Cornus florida.*.

No plant part was ingested that is known to contain **coumarins** (App. II.2).

Lignans and associated compounds

The lignans, syringaresinolmono- to tetrasyccharides, along with some of their degradation products, two new lyoresinoldiglucosides, vomifoliolapioglucoside (a ionon-derivative) and the neolignan icariside-E_5 were isolated in amounts of 20-140 ppm from the stem bark of *A. julibrissin* (Hegnauer, XIb-1, 1996; Fig. II.2, p. 234). Histamine and related imidazole compounds were not found in *A. julibrissin* (Mazzanti *et al.*, 1983), while pyridoxine glycosides with some neurotoxic and arrhythmic activity could be isolated (Higuchi *et al.*, 1992b; App. II.2). The crude methanol extract containing pyridoxine and syringaresinol glycosides exhibited a negative inotropic effect that fits well with the medicinal use as a tonic (see also "saponins"; Kinjo *et al.*, 1991a; Higuchi *et al.*, 1992b). Furthermore, syringaresinol diglucoside is reported to act as a calcium channel antagonist (Higuchi *et al.*, 1992b). In this context, it is remarkable that the bark of *Liriodendron tulipifera* also contains syringaresinol lignans and has a similar reputation as a tonic, but was neither consumed by the focal animals (App. II.2). However, it cannot yet be determined, whether the avoidance of bark from both plant species is caused by the syringaresinol glycosides or other compounds such as the cardioactive compound and coumarins in the bark of *L. tulipifera*, and the pyridoxine glycosides and saponins in the bark of *A. julibrissin*.

Iridoids and secoiridoids, and essential oils

The group of **iridoids and secoiridoids** appears to be completely avoided by the focal lemurs. The subclass Cornidae – which contains the orders Cornales (Cornaceae, Nyssaceae), Dipsacales (Caprifoliaceae), Ericales (Ericaceae) and Oleales (Oleaceae) - is characterised by the rich occurrence of iridoid and / or secoiridoid compounds, and a close chemotaxonomical relationship is explicitly discussed between Caprifoliaceae, Cornaceae and Nyssaceae, and between Oleaceae[1] and Cornales (Frohne and Jensen, 1992; Hegnauer, 2001). Following this chemotaxonomical relationship, nearly all refused plant species can be found in these families and no plant tissue was eaten by the sifakas for which the occurrence of iridoids or secoiridoids was reported.

The only exception to this "species concept" is *Oxydendrum arboreum* (Ericaceae, Cornidae). While the sour tasting leaves were tasted by the sifakas, the bark was vigorously eaten. The

[1] Salidroside (4-hydroxyphenyl-ethanoyl-glucoside) is characteristic of the Oleaceae and precursor of the Cornus-quinol-glucoside and its products.

leaves were used in traditional human medicine as a tonic, a diuretic, and as refrigerant for fevers (App. II.2), and neither for the leaves, nor for the bark the occurrence of iridoid and secoiridoid compounds was explicitly described (App. II.2).

The leaves of *Cornus florida* were spat out by the focal animals, while the bark was completely ignored, and both plant parts have been described to contain iridoid compounds (App. II.2). In human medicine, the bark was previously utilised as a cinchona substitute for malaria, fevers, and as a tonic stomachic, and was included as monograph in the USP (App. II.2).

The other leaf species spat out were the leaves of *Ligustrum vulgare* (Oleaceae). These leaves are known to contain iridoid alkaloids such as cinchonin, cinchonidin and dihydrocinchonidin (App. II.2). Leaves and twigs of *Nyssa sylvatica* (Nyssaceae) are known to lead iridoids similar to those of *Cornus florida*, and the tree remained untouched by the focal lemurs.

Although iridoids occur in the genus *Viburnum* (Caprifoliaceae), they are not explicitly documented to occur in the bark or leaves of *Viburnum prunifolium*. Instead, arbutin as antibacterial phenolic glycoside is mentioned. However, the astringent bark (Viburni prunifolii cortex) has been medicinally used by humans, yet neither the bark, nor leaves were ever ingested by the animals; instead, the entire plant remained untouched. Leaves of *Viburnum rafinesquianum* were even spat out.

The leaves of *Fraxinus americana* were only tasted, *F. pennsylvanica* remained untouched. Although the leaves of *F. americana* were only reported to contain flavonoids, based on the history of the plant family and genus *Fraxinus* iridoid compounds can be expected and may be the underlying reason why their foliage was not chosen. Perhaps due to the fact that the tree grew next to the holding cage in NHE-1, leaves were occasionally tasted.

Therefore, it may be concluded that plant parts leading iridoid or secoiridoid compounds were largely avoided and the pharmacological effects inherent to them such as anti-malaria and febrifuge properties were not required by the focal animals.

Essential oils were ingested to some degree as evidenced by the consumption of leaves from *Liriodendron tulipifera* (Magnoliaceae) and *Liquidambar styraciflua* (Hamamelidaceae), yet there is no hint that plants were selected because of their essential oils contents (compare App. II.2).

Essential oils and oleoresins are characteristic of the gymnosperm families Cupressaceae and Pinaceae, order Pinales. The oil of turpentine of various *Pinus* species is medicinally used by humans. While *Juniperus virginiana* (Cupressaceae) was only passed by the sifakas, the dead brown needles of *Pinus taeda* (Pinaceae) were occasionally ingested. It appears that the essential oils of the gymnosperms were not of interest to the animals in this study. Perhaps the fibre of the *Pinus* needles made them desirable, yet this option has not been investigated.

Bitter tasting compounds (quassinoids[1])

Quassinoids, the bitter tasting principle of the Simaroubaceae, presents an unfavourable group of compounds for the sifakas. *Ailanthus altissima* (Simaroubaceae) was only passed by the focal animals. The bitter tasting quassinoids within the leaves of *A. altissima* presumably overruled the simultaneously present flavonoids and hydrolysable tannins. In traditional human medicine, the bark and root bark were used as a nerval tonic and as an antidiarrhoeal and anthelminthic (App. II.2). Quassinoid compounds revealed antiprotozoal activity with possible anti-malarial effects (App. II.2). The non-usage of the bark corroborates the

[1] Most quassinoids are decanortriterpenes (C_{20}-quassinoids), but also C_{18}-, C_{19}- and C_{25}-quassinoids occur. They are the bitter tasting compounds within the Simaroubaceae (Frohne and Jensen, 1992).

hypothesis that animals did not seek compounds with anthelmintic or antiprotozoal effects to prevent diarrhoea.

Alkaloids

Alkaloids were obviously tolerated to a limited extent, presumably depending on the type and content. Examples give the leaves of *Acer rubrum* and *Liriodendron tulipifera*, which have been eaten by the focal lemurs (App. II.2). On the contrary, the bark of *L. tulipifera*, also containing alkaloids, was not selected by the sifakas. Previously, the bark was medicinally used as a tonic and a stomachic, for rheumatism and fever, and was included in the U.S. Pharmacopoeia. Perhaps other contents such as coumarins and a cardioactive compound (presumably sesquiterpene lactones) in the bark may have a negative impact in regard to sifakas showing a feeding preference.

In Madagascar, *Albizia lebbeck* and *A. gummifera* were utilised as food plants by *P. tattersalli* (Meyers, 1993). These species are known to contain spermine alkaloids in some of their plant parts showing e.g. antibacterial effects (Misra *et al.*, 1995; Rukunga and Waterman, 1996a; for details App. II.2), yet it is unclear which plant parts were ingested by the sifakas. During a study on free-ranging *P. v. coquereli* in Madagascar, no discrimination was apparent between leaf species containing or not containing alkaloids (Ganzhorn and Abraham, 1991), which corroborates the findings that a "certain tolerance" of alkaloids was observed in the focal lemurs. However, for a final correlation between plant tissue ingested and the type and contents of alkaloids contained, further investigation is needed.

Cyanogenic compounds

The Rosaceae are an interesting family to assess the group of cyanogenic compounds. Species of both subfamilies, *Photinia glabra* (Maloideae) and *Prunus serotina* (Prunoideae) contain cyanogenic compounds within their leaves, but only those of *Photinia glabra* were eaten by the study animals. In addition, the leaves of *Liriodendron tulipifera* (Magnoliaceae) were ingested, and are known to contain substantial amounts of cyanogenic compounds with seasonally fluctuating levels (App. II.2). So, it can be concluded that cyanogenic compounds are tolerated by the sifakas and are not the reason for the avoidance of these plant tissues. Either animals possess a highly effective detoxification system, or, together with the feeding behaviour of the golden bamboo lemur (*Hapalemur aureus*) (Glander *et al.*, 1989), it can be hypothesised that these lemur species utilise cyanogenic compounds as source of nitrogen. Conversely, the entire tree *Prunus serotina* remained untouched by the focal lemurs. In previous human medicine, the bark was used against hepatitis, cirrhosis, fever, and circulatory disorders and was listed as officinal drug (USP). A syrup obtained from the bark was commonly used as a cough medicine. Perhaps it is due to the co-occurrence of coumarins and the contents of condensed tannins (App. II.2) that resulted in ignorance of the bark.

Saponins

The evaluation of saponins in regard to food selection remains problematic. Albiziae cortex and Albiziae flos from *A. julibrissin* are officinal drugs described in the Chinese Pharmacopoeia with saponins as the leading compounds within the bark (Stöger *et al.*, 2001). Extensive analytical work has already been dedicated to the stem bark of *A. julibrissin* (App. II.2). According to the Chinese Pharmacopoeia, bark and flowers are used as solving and sedative agents, against restlessness, agitation, sleeplessness and despondency. Moreover, the bark is used against ulcerations in the "orbis pulmonalis" and externally against ulcers, swellings, and injuries from falls and strikes.

The saponin fraction of the bark contains mono-, bis- and tridesmosides and revealed a strong uterotonic activity by stimulating uterine muscle contractions. Furthermore, cytotoxic effects with julibroside III as a highly potent compound (see Fig. II.1, p. 233), and anti-tumour effects were found for several saponins. In part, oligosaccharide moieties were identified as enhancing structures (Ikeda *et al.*, 1995b; Ikeda *et al.*, 1997; Zou *et al.*, 2000a; 2000d; 2005a; 2005b; App. II.2). Basic structures of the triterpenoid sapogenols are machaerinic acid and acacic acid lactone. The genuine saponin glycosides julibroside A_1 to A_4, B_1 and C_1 were gained in 2-15 ppm from the bark of *A. julibrissin*.

While antipyretic, anodyne, sedative, and diuretic effects may be due to the saponin glycosides, syringaresinol diglucoside (see also "lignans") is made responsible for the general use as a tonic in China and Japan "to ease the mind and calm the nerves" (Kinjo *et al.*, 1991a, b; Fig. II.2, p. 234). In a recent study, for an aqueous extract of the stem bark anxiolytic-like effects were described (Kim *et al.*, 2004).

According to the DULC staff (pers. comm.), also flowers and seeds of *A. julibrissin* have been ingested by the Coquerel's sifakas, yet the bark was never been observed to be eaten. However, during the study periods for this project, only the leaves were available and of interest to the animals. Therefore, the leaves will be subject of extensive chemical investigations within this work, for which up to date only scarce information is available.

However, it is reasonable to assume that saponins are tolerated to a limited extent, as they are widely distributed within the plant kingdom. In regard to additional plant species containing saponins that were ingested by the focal animals, saponins are reported to occur in the leaves of *Acer rubrum* and *Photinia glabra* (App. II.2). Although the rate of resorption for saponins from the gastrointestinal tract is generally low, it varies significantly between animal species (Steinegger and Hänsel, 1988). In part, saponins enhance the absorption of other compounds from the intestinal tract (Steinegger and Hänsel, 1988), but in the case of the sifakas, no information is available on the metabolism within the gut system.

With respect to free-ranging *Propithecus* species in Madagascar, four *Albizia* species (*gummifera*, *boivini*, *gaubertiana* and *lebbeck*) are reported to be used by *P. tattersalli* (Meyers, 1993), while leaves of *Albizia greveana* are selected by *P. verreauxi* at Morondava (Ganzhorn, pers. comm.). An unspecified *Albizia* species was a food plant of free ranging *P. v. coquereli* at Ampijoroa (Ganzhorn and Abraham, 1991).

A literature review on *Albizia lebbeck* revealed saponins to occur in nearly all plant organs: roots, heartwood, bark, leaves, flower, pods and seeds (Hegnauer, XIb-1, 1996; Pal *et al.*, 1995). Isolated saponins showed biological effects on germination and growth of agricultural crops (Hegnauer, XIb-1, 1996). Saponins contained in the seeds, exhibited antiovulatory properties (Pal *et al.*, 1995), and various drugs are obtained from *A. lebbeck* for human use (Hegnauer, XIb-1, 1996). For example, saponins from the bark of *A. gummifera* exhibited oxytocin-like activities (albitocines). In Uganda, the bark is used to accelerate the actual birthing process (Hegnauer, XIb-1, 1996).

Thus, it can be inferred that triterpenoid saponins are ingested to some degree, since they are known to occur in several plant organs of *Albizia*. However, it remains to be hypothesised, whether the saponins and their pharmacological effects have lead to the avoidance of *A. julibrissin* bark or whether this was due to other plant compounds (compare "lignans").

Polysaccharides

Plant polysaccharides build another group of chemical compounds which became important during the course of this study. Although gummosis widely occurs in the genus *Albizia*, within the Mimosaceae even more often than within the Caesalpiniaceae (Hegnauer, XIa, 1994), the occurrence of high molecular weight polysaccharides in the leaves of *A. julibrissin* has not been explicitly reported until now, but was detected within this study (see chapter V). The only previous indication for the presence of polysaccharides in *A. julibrissin* was by Moon *et al.* (1985), who screened various oriental medicinal plants containing polysaccharides for their antitumor activity, yet the authors failed to mention which plant part contained these polysaccharides. For *A. julibrissin*, Moon *et al.*, found a relative strong antitumor activity.

The Malagasy species *A. lebbeck*, was a food plant utilised by *P. tattersalli* (Meyers, 1993) and produces a gum exudate from the trunk called “siris-gum” (Hegnauer, 1996). However, there are no documented reports that Coquerel’s sifakas obtain gum exudates e.g. by stripping off the bark of any tree species, or to ingest other plant polysaccharides like mucilage, although their tooth comb could be a suitable tool.

This is corroborated by my own observations: the focal lemurs were never observed to strip (trunk) bark off a tree in order to stimulate gum production. For instance, in previous human medicine, the gum exudate of *L. styraciflua*, the “American Styrax”, was obtained by stripping off the trunk bark. The oleoresin was used medicinally for various external and oral applications, with e.g. dysentery as indication. However, it was not used by the animals of this study. This fits with the observation that *R. copallina* bark of the twigs, which sometimes secreted a gluing resin-like exudate, was neither utilised by the focal lemurs.

Free amino acids

It is interesting to note that large amounts of hydroxyprolin occur in the leaves of *Vitis cinerea* (App. II.2). If applied orally, prolin and hydroxyprolin may play an important role in their interaction with tannins (for further discussion see chapter VI).

Lectins

Lectins may be distributed in various leguminous plants (Frohne and Jensen, 1992; Hegnauer, 2001). For instance, lectins were isolated from the inner bark of *Robinia pseudoacacia* (Fabaceae) with seasonal changes in contents and a marked increase during the autumn (Van Damme *et al.*, 1995; Nsimba-Lubaki and Peumans, 1986; Yoshida *et al.*, 1994). While high levels of lectins were found in the seed, lower levels were identified in the flower and root bark, while the leaves indicated no detectable levels (Van Damme *et al.*, 1995), although it was the leaves that were consumed by the focal lemurs. Therefore, lectins may have a negative impact on food choice.

Conclusion

It can be concluded that some secondary plant compounds were favoured and actively sought out by the foraging lemurs, while others were clearly avoided, and a series of secondary plant compounds may be tolerated to some extent, presumably depending on dosage and implicated pharmacological effects. Simultaneously present chemical compounds may overrule other less favoured compounds and vice versa, but not in any case it becomes visible as to why the focal lemurs treated the plant tissue the way they did. When leaves were spat out, they undoubtedly contained chemical compounds that exceeded the tolerance levels of the study animals. Furthermore, it has to be noted that in App. II.2 only available information could be gathered and further chemical investigations on both the quality and quantity of secondary plant compounds would help to recognise further criteria to evaluate the food selection of the sifakas. Both the seasonal fluctuation of plant compounds and individual food preferences and eating habits (see chapter B; Richard 1978a, b), make the evaluation of plant tissue in regard to their palatability, rather problematic. However, the information compiled in App. II.2 is a useful means to recognise favoured and less favoured chemical compounds in regard to food choice and possible implicated beneficial effects for the well-being of the sifakas.

High contents of hydrolysable tannins were obviously sought out by the focal lemurs and can be expected to occur in the leaves of *R. copallina*, the most preferred food plant at the DULC. On the contrary, levels of condensed tannins seem to be minimised. Animals apparently discriminated between hydrolysable and condensed tannins.

The role of *A. julibrissin* leaves, the other highly preferred food species, cannot be conclusively evaluated from the current available literature. Hence, both preferred leaf species, *R. copallina* and *A. julibrissin*, will be extensively investigated within this study as to their secondary leaf compounds (chapters III to V) with the averaged daily intake of these compounds to be discussed in the context of diarrhoea (chapter VI).

Hydroxycinnamic acids are supposed to be ingested inasmuch that they are typically associated with tannins and flavonoids. Essential oils were obviously tolerated, even in larger amounts, while the ingestion of alkaloids appears to be dependent on dosage and inherent pharmacological effects. Saponins are thought to be tolerated at least in small amounts. Coumarins were not proved to be ingested, while unlike expectations, even larger amounts of cyanogenic glycosides are apparently not toxic for the sifakas (as they are for humans), perhaps due to differing metabolic pathways. Yet conversely, plants leading iridoid and secoiridoid compounds were consistently refused in accordance with chemotaxonomical relationships between the species concerned. Likewise, irritant phenolics were also consistently avoided. Some groups of chemical compounds remain both difficult and equivocal in their evaluation, such as naphthoquinones, stilbenes, lignans, the bitter compounds quassinoids and lectins, which were rather avoided by the focal lemurs.

Chemotaxonomical relationships between the plant species within the animals' environment, provided a useful means to assess food preferences and food avoidance, since with only few exceptions, the animals chose their food plants in agreement with chemotaxonomical principles.

Another interesting aspect of this study is the comparison between the medicinal plant use by humans (previously and currently), and the selection or non-selection of these plant species by the focal lemurs (App. II.2), which may coincide or diverge.

In particular, those plants with known antidiarrhoeal and beneficial gastrointestinal effects were observed to be actively selected by the focal lemurs, albeit different plant parts may be used by humans and the sifakas: while in (traditional) human medicine often the barks were used (with predominantly condensed tannins), the sifakas selected the leaves (with predominantly hydrolysable tannins) of these species. Plant parts known as "antidiarrhoeal drugs" within human medicine containing hydrolysable tannins were also selected by the focal lemurs. This corroborates the discrimination between condensed and hydrolysable tannins within the plant tissue ingested. Furthermore, plant organs of species (drugs) with indications such as malaria, fever, antiprotozoal or anthelmintic effects appear to be avoided by the focal animals.

C.III Identification of flavonoids and simple phenolics in the leaves of *Rhus copallina* and *Albizia julibrissin*

1 Introduction

During observations on the feeding behaviour of captive Coquerel's sifakas at the Duke University Lemur Center (chapter B) two major food plants were recorded, *Rhus copallina* and *Albizia julibrissin*, which subsequently, were submitted to extensive chemical investigations. After an extensive literature survey on their secondary plant compounds, gallotannins are expected to be leading compounds in the leaves of *R. copallina*, while scarce information exists on the content of *A. julibrissin* leaves (details see chapter C.II and App. II.2).

Some preliminary assays commenced with the leaves of *R. copallina* and *A. julibrissin* that were harvested during the first observation period in spring with additional plant harvests in July to gain preliminary experimental data as to what major classes of chemical compounds were present. These preliminary assays also included an additional four leaf species that had been offered as browse (Tab. C.1, p. 57). These additional samples were expected to contain some classes of compounds that were in common among the various food plants. Thin-layer chromatography was then used to detect and exclude the classes of compounds occurring within these six leaf species.

For further quantitative analyses and pharmacological evaluations of the major chemical compounds it was inevitable to isolate them preparatively from the leaves of *R. copallina* and *A. julibrissin* and to elucidate their structures by various methods including UV spectroscopy, mass spectrometry and NMR-spectroscopy.

2 Materials and Methods

2.1 Plant material

For origin and identification of the plant material see C.I.2.1.

For preliminary assays, the leaves of *R. copallina* and *A. julibrissin* from the May harvest were used, supplemented with additionally harvested plant material from the July. The other four leaf species included were all harvested in May. For preparative isolations, the leaves of *A. julibrissin* were collected in May while the leaves of *R. copallina* were collected in July.

For preliminary assays the leaf material was ground and sieved according to C.I.2.2.

2.2 Thin layer chromatography (TLC)

Thin layer chromatography was used in preliminary assays to classify and identify different classes of compounds and numerous individual compounds within the leaves of the food plants offered as well as to control various analytical steps.

Quality of the TLC plates used

Most TLC analyses were performed on
TLC aluminium sheets; 20x20 cm; Silica gel 60 F_{254} (Merck); 0.20 mm (layer thickness).

Reversed phase thin layer chromatography was performed on
TLC plates (glass); 20x20 cm; RP-18 F_{254} S (Merck); 0.25 mm (layer thickness).

TLC-solvent-systems to develop TLC plates

The following TLC-systems were used to develop Silica gel aluminium sheets. All solvents were prepared by mixing the corresponding volumes:

System 1: Ethylacetate / methanol / water / acetic acid (50:8:6:1)
System 2: Ethylacetate / methanol / acetic acid / water (60:15:15:10)
System 3: Ethylacetate / methanol / water (100:13.5:10)
System 4: Ethylacetate / ethylmethylketone / formic acid / water (50:30:10:10)
System 5: Ethylformiate / formic acid / water (80:10:10)
System 6: Toluene / methanol (90:10)
System 7: Toluene / methanol / ethylmethylketone / acetylacetone (40:30:20:10)
System 8: Toluene / ethylacetate / diethylamine (70:20:10)
System 9: 1-Butanole / acetic acid / water (40:10:50)
System 10: Dichloromethane /methanol (80:20)
System 11: Dichloromethane /methanol / water (64:50:10)

TLC-systems for Silica gel RP-18 TLC plates (glass):

System 12: Water / methanol / acetic acid (60:40:1)
System 13: Water / methanol / acetic acid (50:50:1)

TLC-systems 1 and 3
were largely used to examine the leading compounds of *R. copallina* and *A. julibrissin* as well as to review various extraction methods and steps needed to isolate and identify individual compounds. That way, different extraction methods based on varying pH (Stas-Otto) and solvent polarity, precipitation experiments, e.g. with lead acetate, and different methods of hydrolysis (methanolysis and TFA-hydrolysis; see C.IV.2.3) were monitored especially for *A. julibrissin* and *R. copallina* leaves. Once the main compounds of *A. julibrissin* and *R. copallina* were recognised to be flavonoid glycosides and tannins, TLC system 3 was replaced by system 1 by adding acetic acid in order to reduce the tailing effect especially of the hydrolysable tannins. Hence, further evaluation and control of preparative extraction steps (by means of a polyamide column and preparative HPLC method) as well as reviews of plant extracts prepared from all food plants for a quantitative determination (by means of a "Bit Beater" with and without subsequent hydrolyses) were monitored in TLC system 1.

TLC-system 2 (Jerga, 1989) and 11
were used to examine the sugar moieties of flavonoid glycosides after methanolysis (Jerga, 1989).

TLC-system 4
was predominantly used to separate flavonoid glycosides. *A. julibrissin* fractions eluting from the preparative HPLC column were examined in system 4 as already described for system 1.

TLC-system 5,
which is like system 4 appropriate to separate flavonoid glycosides, was chosen to examine isolated tannin fractions of *R. copallina* leaves (C.IV.2.5) to check for their purity. That fraction was also investigated in the TLC-systems 3, 11 and 12.

TLC-systems 6 and 7 (Jerga, 1989)
were used to look for flavonoid aglyca. Whereas in system 6 unhydrolysed glycosides do generally not move and the flavonoid aglyca myricetin, quercetin and kaempferol hardly move, more lipophilic compounds can be separated. The TLC-system 7 was revealed to be more appropriate in this study in the need to separate flavonoid glycosides (e.g. of quercetin type) from their corresponding aglyca.

TLC-system 8
was used to search for alkaloids (Stahl and Schild, 1981).

TLC-systems 9 and 11
were used to look for saponins (Stahl and Schild, 1981). Furthermore, system 9 is appropriate to separate flavonoid glycosides (Stahl and Schild, 1981).

TLC-system 10
was used like system 3 to evaluate first extracting experiments based on different solvent polarity.

TLC-systems 12 and 13
were assayed for their separating properties on reversed phase glass plates. System 12 yielded a better resolution compared to system 13 with respect to flavonoid glycosides and tannins.

Apolar, lipophilic solvent systems like 6 and 10 were also useful to search for apolar structures like terpenoid compounds.

Extracts or solved substances were set on the starting point by means of a glass capillary or an appropriate Eppendorff pipette. Most TLC plates were developed over a distance of about 10 cm, but also 6-8 cm led to satisfying results, if only few substance was used. Distances between the points on the starting line were 1.5 cm. Development was performed without solvent saturation in a covered glass chamber of appropriate size.

All plates were first looked at in day-light, then under the UV light at 254 nm and 366 nm and finally after detection with spray reagents in daylight and - if necessary - under UV light at 366 nm (Markham, 1975; Stahl and Schild, 1981).

About 30 ml of solvent was sufficient to develop two TLC plates over a distance of 10 cm, respectively.

Spray reagents

Anisaldehyde / sulphuric acid reagent (= anisaldehyde / H_2SO_4) (Stahl and Schild, 1981)
In the following sequence 0.5 ml anisaldehyde were mixed with 10 ml acetic acid (99-100 %), 85 ml methanol and 5 ml sulphuric acid (95-97 %).

After spraying, plates were heated up to 100-110 °C in an oven for about 5 to 10 minutes until colours were fully developed. However, in some cases it was necessary to heat up even to higher temperatures (e.g. 120 °C or even up to 140 °C). Repeatedly heating can reactivate colours.

The complete reagent cannot be kept for a protracted time. If its colour turns red, it must be discarded. But it is possible to store a mixture of anisaldehyde, acetic acid and methanol for a length of time in the refrigerator. Just before use, sulphuric acid has to be added.

This reagent is appropriate for staining a wide range of natural products (especially terpenoids of essential oils). However, it was universally used as it yielded characteristic colours with most compounds tested.

Thymol / sulphuric acid reagent (= Thymol / H_2SO_4) (Jerga, 1989)
0.5 g Thymol, 95 ml ethanol and 5 ml sulphuric acid (95-97 %) were cautiously mixed. TLC plates were sprayed with a freshly prepared reagent and heated up to 120 °C for about 5 minutes until colours were totally developed. In part it was necessary to heat up to 140 °C.

This reagent is useful to detect monosaccharides, e.g. as sugar moieties of flavonol glycosides after hydrolyses. Furthermore, it is a valuable reagent to stain flavonoid aglyca and glycosides.

Vanillin / sulphuric acid (= Vanillin / H_2SO_4) (Scholz, 1987)
3 g vanillin were solved in 100 ml ethanol, finally 1 ml sulphuric acid (95-97 %) was added. After spraying, TLC plates were heated up to 110 °C for about 5 minutes.

In some cases a second step was incorporated, to spray one reagent over a first reagent. Reagents containing sulphuric acid with subsequent heating were generally suitable to be sprayed over other reagents. For instance, a combination of the $FeCl_3$ reagent with anisaldehyde / H_2SO_4 or vanillin / H_2SO_4 was possible and revealed to be useful to detect first tannins and afterwards additional compounds, especially when lying under the tannin fraction.

Diphenylboryloxyethylamine reagent = “Reagent A for natural products according to Neu” (Jerga, 1989)
Solution A: 1 % (m/v) solution of diphenylboryloxyethylamine in methanol

Solution B: 5 % (m/v) solution of polyethylene glycol 400 in ethanol

The dried plate was first sprayed with solution A and then with solution B. The evaluation was performed after 30 min. in day light and under UV light at 366 nm.

The solution of polyethylene glycol (PEG) acts as moistening agent and preserves the colours. It is especially appropriate to detect α- and γ-pyrones (flavonoids).

Ferric chloride reagent ($FeCl_3$ reagent)
A solution of 10 % (m/v) $FeCl_3$ in methanol was provided for the following spray reagent: 0.5 ml of that solution had to be diluted with 19.5 ml methanol, respectively.

$FeCl_3$ yields coloured complexes with phenolic compounds and hydroxamic acids. In case of gallic acid and gallic acid derivatives (gallotannins), grey-blue coloured complexes could be detected on TLC plates.

Some drops of the 10 % methanolic $FeCl_3$-solution are sufficient to test directly for hydrolysable tannins in solution yielding ink-blue to black colours. Condensed tannins yield green coloured complexes (Wagner, 1999).

Molybdenum-phosphoric acid (DAB 9)
4 g molybdenum-phosphoric acid were dissolved in 40 ml water; 60 ml sulphuric acid (95-97 %) were added while cooling the solution.

It is a universal reagent for reducing compounds.

Iodine vapour (Markham, 1975; Stahl and Schild, 1981)
Some crystalline metallic iodine (10-20 g) was put into a glass chamber appropriate for developing TLC plates. The dry and already developed TLC plate was set into that chamber to be detected by iodine vapour.

The advantage of this reagent is that compounds, which do not extinguish under UV light, become visible by iodine vapour. But the colour fades after a while as iodine vaporises. Universal reagent.

Dragendorff reagent (according to Puech) (Stahl and Schild, 1981)

The basic solution consisted of the following ingredients which was placed in a brown bottle: 1.7 g basic bismuth nitrate and 20 g tartaric acid were dissolved in 40 ml water. This solution was mixed with a solution of 16 g potassium iodide in 40 ml water and stirred for one hour. Finally, the resulting solution was filtered.

The actual spraying reagent has to be freshly prepared each time:
5 ml of the basic solution have to be added to 15 ml water. Afterwards, a newly provided solution of 0.1 % (m/v) Na NO_2 in water has to be sprayed over in a second step.

This reagent is appropriate to detect alkaloids.

2.3 Test to discern hydrolysable from condensed tannins

2.5 g powdered plant material were extracted with 40 ml water for 20 min. at 50 °C. 1 g sodium acetate was added to the warm extract. The suspension was left to cool down and filtered. The residue was rinsed with 10 ml water, and filtrates were unified and filled up to 50 ml with water. 25 ml of that solution were refluxed for 30 min. with 2.5 ml conc. hydrochloric acid (36 %) and 5 ml formaldehyde (35 %), cooled down at room temperature and filtered.

While condensed tannins yield a precipitation of red coloured polymers (phlobaphenes) in formaldehyde-hydrochloric acid solution, hydrolysable tannins stay in solution. The latter yield blue coloured complexes with ferric chloride in alcoholic solution (reagent see above), while condensed tannins give green colours (Steinegger and Hänsel, 1988; Wagner, 1999).

A. julibrissin could not be extracted in that manner because of the mucilage contained in the leaves, therefore 50 % (v/v) aqueous methanol was used. The plant material was first dispersed in methanol and afterwards, the same volume water was added. Otherwise the fraction of soaked mucilage prevented every attempt of filtering the solution.

2.4 Polyamide column chromatography and HPLC

Preparation of the plant extracts

Preparative isolation of individual leaf compounds only concerned *R. copallina* (harvested in July) and *A. julibrissin* (harvested in May).

Approximately 15 g of either leaf material was used, respectively. Leaflets had been removed previously from the stems being attached to as described in C.II.2.2. Furthermore, leaf buds at the tip of *A. julibrissin* stems, which had not always been ingested by the study animals in spring, were removed as well.

R. copallina leaves were first ground by means of a mortar and pistil and then pre-extracted with n-hexan (about 100 ml) to remove highly unpolar and lipophilic compounds, e.g. chlorophyll. However, before discarding the n-hexan fraction, it was checked by TLC for its spectrum of compounds to prevent any loss of possibly valuable components. Solvent residues of n-hexan were subsequently evaporated (Rotavapor®).

The further extraction of *R. copallina* leaves was performed similar to the extraction of *A. julibrissin* leaves with 150 ml aqueous methanol (80 % v/v). The dispersed plant material was ground for several minutes in intervals by means of an Ultra-Turrax homogeniser (Ultra-Turrax T 25, Janke und Kunkel, IKA-Labortechnik). The Ultra-Turrax was cleansed with some millilitre of pure methanol which was then added to the extract. During this time the suspension was cooled with ice. Then, the suspension was stirred in a covered flask at room temperature for about 1.5 hours (magnetic stirrer). The mixture was filtered under low pressure using a water jet pump and the remaining plant material was rinsed with some millilitre of 80 % (v/v) methanol. The extract was concentrated *in vacuo* (Rotavapor®) at low temperature (30-40 °C, water bath). The residue was dispersed in some millilitre 80 % (v/v) methanol (q.s.) and centrifuged for a couple of minutes. The supernatant solution was used for further separation on a polyamide column.

Comment:
80 % (v/v) aqueous methanol accounted best for the diversity of hydrophilic and lipophilic secondary plant compounds while a certain reduction of highly lipophilic compounds as chlorophyll could be achieved.

Except for a pre-extraction of *R. copallina* leaf material with n-hexan no other pre-treatment was found to be necessary. A pre-extraction by means of a LiChrolut® column did not achieve any additional surplus in case of subsequent use of a polyamide column with respect to a better resolution.

Separation by means of a polyamide column

The concentrated plant extract of either plant was put onto the column and the eluents were selected from the hydrophilic, polar end to less polar properties, terminating with methanolic ammonia.

Fractionation was checked by means of an Uvicord (Uvicord S II, Pharmacia LKB for peak recognition associated with REC 101, Pharmacia LKB as recorder) at 254 nm. The fractions gained were first reviewed by TLC analytic (solvent system 1 and “Naturstoff”-reagent and anisaldehyde / H_2SO_4 as spraying reagents) and then by analytical HPLC analyses (C.IV.2.1). Finally it was determined, which fractions had to be taken, which should be unified and which were discarded.

The first fractions eluted with water, contained predominantly polysaccharides, giving blue fluorescence at 366 nm and staying at the starting position in the solvent system 1. They were not used for further separations. Fractions with too low amounts of any substance to be isolated or that were too impure for subsequent elucidation of the chemical structure were discarded as well.

Every fraction used for further separation on the preparative HPLC column was concentrated in vacuo (30-40 °C water bath) and dissolved in few ml of 80 % (v/v) aqueous methanol.

The following eluents were used to fractionate the aqueous plant extracts put onto the column:

de-ionised water (Millipore®); 5 %, 10 %, 30 %, 50 % and 60 % aqueous methanol; pure methanol (100 %); EtOAc/MeOH 70 %; pure EtOAc (100 %), 0.1 % methanolic NH_3. All eluents were prepared by mixing the corresponding volumes.

Size and volume of the glass column
44 cm in height; 4.4 cm in diameter; comprising a volume of about 670 ml.

Polyamide material
MN - Polyamide SC 6 (purchased from Macherey and Nagel); particle size 0.05-0.16.

Activation / reactivation of the polyamide
The polyamide material was dispersed in Millipore® water several times and the fines were decanted each time, until the supernatant solution was clear.

Then the polyamide material was poured into the glass column.

To activate or reactivate the material, it was first treated with 10 % aqueous NH_4OH, washed with water until neutral pH, then treated with 10 % aqueous HCl and finally washed neutral again. Alternatively, 1 N NaOH could be used instead of the ammonium solution to reactivate the material.

Preparative HPLC separation

The solvent system formic acid / methanol was used for every separation procedure. Every fraction eluted from the preparative HPLC column was checked by analytical HPLC procedure (C.IV.2.1) for its purity and then fractionated again preparatively if needed. Finally, every fraction was also reviewed by TLC analysis, using solvent system 1. Pure fractions were concentrated again, stored in Eppendorf tubes and brought to near dryness by means of a Speed Vac centrifuge. These isolated compounds were subsequently identified and elucidated by their UV spectra, mass spectrometry and NMR spectroscopy. (UV spectra were obtained by means of the photodiode array detector during the analytical HPLC procedure to verify the substance's purity, see C.IV.2.1).

The preparative HPLC separation was performed on a Liquid Chromatograph (System Gold, Beckman Instruments, USA) as reversed phase chromatography on a Nucleoprep® 100-10 C_{18} column (Macherey-Nagel, VP 250 / 40 mm). A 136 Pump and a 166 Detector were used. UV detection of the eluting fractions was performed between 260 and 280 nm. (Fractions gained from *A. julibrissin* leaves were detected at 260 nm, while fractions from *R. copallina* leaves were detected at 270 to 280 nm.)

Solvent system and gradient

Solvent A: 1 ‰ aqueous formic acid (pH 3);

Solvent B: methanol.

The following gradient, at a flow rate of 12 ml / min., was used:
0-3 min. linear gradient to 90 % A; 3-13 min. linear gradient to 60 % A; 13-113 min. 60 % A; 113-120 min. linear gradient to 10 % A; 120-130 min. 10 % A; 130-150 min. linear gradient to 100 % A, which was held constant to 200 min.

For some compounds eluting from the column it was favourable to hold the solvent composition manually constant for some minutes before continuing the programmed gradient.

2.5 Structure identification

Mass spectrometry

Most mass spectrometric experiments were performed at the IPB (Halle / Saale), supplemented with additional experiments at the University of Braunschweig and at the University of Bremen.

ESI-MS

Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV (positive ions); 3.5 kV (negative ions); heated capillary temperature 200 °C; sheath gas and auxiliary gas: nitrogen coupled with a syringe pump (HARVARD Apparatus); and
Esquire-LC, Bruker Daltonik, capillary voltage ± 3.8 kV, dry gas (nitrogen) temperature 300 °C, direct inlet via syringe pump (Cole-Parmer), flow rate 2 µl / min., solvent: methanol / DMSO (100:1).

Comment**:**
A priori, ESI-MS is the most soft ionisation technique in mass spectrometry, which predominantly yields quasimolecular cations or anions, depending on polarity of the ESI mode. However, to some extent fragmentation is inducible and is evidenced in some described spectra (App. II.3). Normally, both polarities were measured although in most cases only the

negative mode contained valuable information, which is in accordance with the findings of Gioacchini *et al.* (1996) on phenolic compounds.

EI-MS
AMD 402 (AMD Intectra GmbH), ionisation energy 70 eV (direct inlet system); source temperature 225 °C; and
Finnigan MAT 8200, ionisation energy 70 eV (direct inlet system); source temperature 200 °C.

Comment:
All isolated fractions gained from *R. copallina* and *A. julibrissin* leaves were measured with EI-MS, whether a glycoside or an aglycone was isolated. In case of flavonoid glycosides, the aglyca were released thermally under EI-conditions and were hence detected as such. So, electron impact spectra normally show only the corresponding aglycone. Masses of fragments, which occurred in the EI spectrum but could not be assigned to any reasonable fragment, were listed in App. II.3 without interpretation.

FAB-MS
Finnigan MAT 8200; collision gas: xenon; 8 keV; matrix: glycerol.

DCI-MS
Finnigan MAT 8200; reactant gas: NH_3; heating rate 8 mA/s; source temperature 200 °C.

HR-MS
Finnigan MAT 8200 MS using electron impact ionisation; resolution 10 000 (10 %-valley-definition).

Detailed data of mass spectrometric experiments are presented in App. II.3.

GC-MS
GC-MS analyses were performed on hydrolysed plant extracts after methanolysis (Jerga, 1989; see C.IV.2.3).

25 µl of each hydrolysed sample were solved in 100 µl methanol. One third was taken, brought to dryness and 50 µl MSTFA were added. The mixture was heated approximately one hour up to 80 °C and injected into the GC apparatus.

GC-conditions: DB1-30 W, 100 kPa helium, injector 250 °C, oven 150-320 °C, 10 °C per minute, detector FID 320 °C.

EI-MS: Finnigan 4500, ionisation energy 70 eV, source temperature 200 °C.

NMR-spectroscopy

Nearly all NMR spectra were measured on a Bruker AM-400 or a DRX-400 spectrometer: frequency for ^{1}H: 400.1 MHz; frequency for ^{13}C: 100.6 MHz. Some additional spectra were measured on a Bruker AM 360 (frequency for ^{1}H: 360 MHz; for ^{13}C: 90 MHz). For one sample the ^{13}C-spectrum was measured at 150 MHz.
Solvent and chemical shift reference: DMSO-d_6, delta (H) = 2.50; delta (C) = 39.50.

Chemical shifts are given in ppm, coupling constants (J-values) in Hertz (Hz). Generally, DMSO-d_6 was used as solvent; if other solvents were used such as methanol-d_4 or D_2O, this is indicated in App. II.3.

Parameters for ^{1}H NMR-spectroscopy

number of scans	24
spectral width	8064.5 Hz
number of data points	32 K

Parameters for ^{13}C NMR-spectroscopy

number of scans	200 to several thousand
spectral width	25000 Hz
number of data points	64 K

All ^{13}C spectra were measured with proton decoupling.

DEPT

In edited spectra, CH_3- and CH- groups result in a positive peak, while CH_2- groups yield a negative peak. Quaternary carbons are not visible.

If a ^{13}C spectrum was measured, always a DEPT experiment was performed to aid in the assignment.

2.6 Chemicals and reference compounds

Reference compounds used for qualitative TLC and HPLC analyses

(from the stock of the "Institut für Pharmazeutische Biologie", Braunschweig)

caffeic acid	galactose	hamamelitannin	saponin (Merck)
chinidin sulphate	galacturonic acid	hyperoside	xylose
chlorogenic acid	glucose	pyrogallol	
coffein	glucuronic acid	rhamnose	
ellagic acid	glycyrrhetinic acid	rutin	

Reference substances used for TLC analyses and as standards for qualitative and quantitative HPLC analyses

Myricetin (Fluka) [529-44-2]	Cannabiscetin; 3,3',4',5,5',7-Hexahydroxyflavone Purity: ~ 97 % (HPLC)
Quercetin (Merck) [6151-25-3]	3,3',4',5,7-Pentahydroxyflavone-2-(3',4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one Purity: ≥ 98 % (HPLC)
Kaempferol (Fluka) [520-18-3]	Robigenin; 3,4',5,7-Tetrahydroxyflavone Purity: ≥ 96 % (HPLC); H_2O ≤ 5 %
Quercitrin (Roth) [522-12-3]	Quercetin-3-L-rhamnoside; 3,3',4', 5,7-Pentahydroxyflavone-3-L-rhamnopyranoside Purity according to the statement of the company: ≥ 98 % (HPLC)
Gallic acid (Roth) [149-91-7]	Gallic acid monohydrate; 3,4,5-Trihydroxybenzoic acid hydrate Purity: ≥ 98 % (HPLC)
Tannic acid[1] (Fluka) [72401-53-7]	Gallotannin, Tannin (DAB), Tanninum (Ph.Helv.) synonym: Acidum tannicum

[1] Tanninum (Ph.Helv.), tannin (ÖAB), tannic acid (USP), Acidum tannicum (lat.).
According to USP "tannic acid is a tannin usually obtained from nutgalls, the excrescences produced on the young twigs of *Quercus infectoria* Oliver, and allied species of *Quercus* Linné (Fam. Fagaceae), from the seed pods of Tara (*Caesalpinia spinosa*), or from the nutgalls or leaves of sumac (any of a genus *Rhus*)."
According to European Pharmacopoea (ÖAB, Ph.Helv.) tannin, a yellowish-white to light brownish powder, is extracted from nutgalls, Gallae (Galla ÖAB). (Gallae are excrescences which are induced by insects.) Various *Rhus* species are the source of Chinese Gallae, e.g. *Rhus sinensis*, while Turkish Gallae originate from *Quercus infectoria* (Rimpler, 1999).
According to Doorenbos and Box (1976) commercial gallotannins are obtained from the leaves of *Rhus coriaria* (native to Spain and Sicily), and from leaf galls of *Rhus semialata* growing in China and Korea. During the search for sources of tannins for industrial purposes originating from the USA analyses were extended to *Rhus glabra* and *Rhus copallina*, which both were shown to contain gallotannins of USP quality

Following reference compounds were generously supplied

1,2,3,6-tetragalloylglucose, 1,2,3,4,6-pentagalloylglucose	Prof. Dr. G. G. Gross, University of Ulm, Germany
julibroside III (Fig. II.1, p. 233) syringaresinol diglucoside (= liriodendrin) (Fig. II.2, p.234)	Prof. Dr. J. Kinjo, faculty of Pharmaceutical sciences, Kumamoto University, Japan
tannin albuminate[1]	Knoll Deutschland GmbH, BASF Pharma, Germany, (now Dr. Rentschler Arzneimittel GmbH)
Plantaginis ovatae testa (Ispaghula husk, DAC 1986)	Dr. Falk Pharma GmbH, Germany

Solvents
For TLC analyses, solvents like methanol, ethylacetate and dichlormethane were distilled before use. Solvents used for preparative isolations or quantitative determinations were of p.a. or HPLC grade.

3 Results and discussion

3.1 Preliminary assays

Thin layer chromatography was an extensively used method to get first information on the classes of chemical compounds and in a second step, on individual compounds occurring in the leaves. Different solvent systems combined with varying spray reagents allowed for an effective and fast screening of a large number of compounds. HPLC runs with photo diode array detection of UV spectra between 210 and 450 nm supported and verified first investigations (C.IV.2.1). The effectiveness and simple handling of thin-layer chromatography shall encourage field workers to use this method to screen food plants and non food plants for their secondary plant compounds.

In this study, the role of saponins will be illuminated first. As triterpenoid saponin glycosides build the characteristic compounds of the bark of *A. julibrissin* (App. II.2), they could principally be expected to occur in the leaves. Methanol extracts of *A. julibrissin* leaves gave no hint of saponins (TLC-systems 5, 9 and 11 combined with spray reagents like anisaldehyde / H_2SO_4 and vanillin / H_2SO_4; reference compounds: standard saponin (saponinum album) and glycyrrhetinic acid).
The foam test (Steinegger and Hensel, 1988; Stöger *et al.*, 2001) was performed on all six species harvested in the spring (see Tab. C.1, p. 57), but results remained ambiguous.

Julibroside III (for structure see Fig. II.1, p. 233), which had been isolated from the stem bark of *A. julibrissin* (Ikeda *et al.*, 1995a), was used as a reference compound for analytical HPLC runs. Although julibroside III should occur in the leaves (Kinjo, pers. comm.), it remained difficult to detect this substance by HPLC analyses in *A. julibrissin* leaves. If at all, only trace

(Doorenbos, 1976; Doorenbos and Box, 1976).
Tannin is a mixture of isomeric and homologous gallotannins with penta- to nonagalloylglucoses as main components. Chinese tannins derive from 1,2,3,4,6-penta-O-galloyl-ß-D-glucose. In contrast, Turkish tannins derive from two basic structures, the tetra-O-galloyl- and penta-O-galloyl-ß-D-glucose, and contain hexahydroxydiphenic acid in addition (Rimpler, 1999; Wagner, 1999).

[1] The remedy Tannalbin® for the treatment of diarrhoea consists of an 1:1 tannin : protein complex (= Albumini tannas, tannin albuminate).

amounts could be detected. As saponins do apparently not constitute the major class of compounds in the leaves of *A. julibrissin*, no further attempts were undertaken for their determination.

Syringaresinol diglucoside (also called liriodendrin; for structure see Fig. II.2, p. 234), is another important substance known to occur in the bark and was used as a reference compound. It could not be detected in *A. julibrissin* leaves by HPLC analyses in this study.

Thin layer chromatography was used as means to search for alkaloids in *R. copallina* and *A. julibrissin* leaves (TLC-system 8, Dragendorff spray reagent, iodine vapour), but no alkaloids could be detected.

Most coumarins yield fluorescing spots on TLC plates. As no fluorescing spots could be discerned, a large number of coumarins were excluded and were not presumed to occur in *R. copallina* or *A. julibrissin* leaves.

Searching for flavonoid glycosides in the food plants was a promising and successful approach to get determinant compounds (TLC-systems 1, 2, 3, 4, 12, 13; staining reagents: diphenylboryloxyethylamine reagent; thymol / H_2SO_4; and especially anisaldehyde / H_2SO_4). Quercitrin and hyperoside have already been described to occur as flavonol glycosides within *A. julibrissin*, but the plant organ of their occurrence was not specified (Kaneta *et al*., 1980; App. II.2). Furthermore, 3', 4', 7-trihydroxyflavone has been shown to occur in the stem bark, while cyanidin-3-monoglucoside was revealed to occur in the flower of *A. julibrissin* (App. II.2). Screening on TLC plates and later verification by HPLC analysis revealed that quercitrin occurred in many of the food plants and constituted the main flavonol glycoside in *A. julibrissin* leaves. *R. pseudoacacia* leaves (system 1, anisaldehyde / H_2SO_4) showed the most deviant pattern of flavonoids on TLC plates of all food plants tested.

Screening for free occurring aglyca such as quercetin, myricetin and kaempferol (TLC-systems 1, 2, 3, 6 and 12; spray reagents like for glycosides) remained negative. Therefore, the *A. julibrissin* leaves gathered in May were hydrolysed (Jerga, 1989) and subsequently analysed by GC-MS. Quercetin, myricetin and kaempferol were found as aglyca with myricetin detected, if at all, in only tiny amounts[1] (details see chapter IV). Hence, these three aglyca were used as reference compounds for further quantification assays of flavonol aglyca after hydrolysis. Furthermore, the flavone acacetin was detected in *R. pseudoacacia* leaves by GC-MS analyses.

Separations based on different pH (e.g. Stas-Otto separation, Pharmacopoeia-DDR) and solvent polarity (sequential solvent extraction with solvents of varying polarity, and partitioning between water and an organic phase like n-butanol, dichlormethane etc.) did not give any hint to expect any highly lipophilic compounds like essential oils in *R. copallina* or *A. julibrissin* leaves (TLC-system 3, 6, 10, 12, 13 combined with various spray reagents, especially anisaldehyde/ H_2SO_4). During this screening, 80 % (v/v) methanol exhibited best extracting properties for *R. copallina* and *A. julibrissin* leaves, which was thereafter used for all further qualitative and quantitative analyses.

Several assays were performed to characterise the group of tannins that occurred in the food plants with special respect to *R. copallina* leaves. For various TLC and subsequent HPLC analyses commercial tannin (Acidum tannicum, tannic acid), gallic acid, ellagic acid, tetragalloylglucose and pentagalloylglucose were used as reference compounds. On TLC plates the fraction of gallotannins could be detected with ferric chloride reagent yielding grey-blue colours.

[1] It has to be noted that the appearance of aglyca in the GC chromatogram (kaempferol, quercetin, myricetin) is reverse compared with the HPLC chromatogram (myricetin, quercetin, kaempferol).

The test to discern hydrolysable from condensed tannins remained in part ambiguous. Except for *A. julibrissin* leaves all plant samples (*R. copallina*, *R. pseudoacacia*, *L. styraciflua*, *C. canadensis*, *A. rubrum*) including commercial tannic acid produced a red to reddish brown precipitation after treating the aqueous extracts with mineral acid and formaldehyde, although this should only occur in case of condensed tannins (Steinegger and Hänsel, 1988; Rimpler, 1999; Wagner, 1999). Testing the filtrated solution for tannins with alcoholic ferric chloride reagent produced a range of mixed colours, which was presumably due to the rich occurrence of flavonoids, as has been shown on TLC plates (TLC-system 1). Best results were gained after separation of the filtrates, and enhanced results utilised hot water extracts, on TLC plates and detection with ferric chloride reagent. Only *R. copallina* and *A. rubrum* leaf extracts yielded unambiguously positive results indicating the presence hydrolysable tannins. However, during this assay, a rich occurrence of plant mucilage was discovered in *A. julibrissin* leaves, which has not been described before. As it was impossible to filter the hot water extract of the leaves[1] without disturbing this assay, this test method had to be modified (C.III.2.3). At this point it is interesting to recall that the same difficulties in filtering aqueous extracts containing mucilage were already described by Bate-Smith (1977).

It can be summarised that by preliminary assays flavonoids and tannins were shown to be the main groups that occurred in *R. copallina* leaves, while flavonoids and the plant mucilage were shown to be the major compounds in *A. julibrissin* leaves. The ubiquitous groups of tannins and flavonoids (including their major aglyca) were also shown to occur in a limited extent in the four other food plants offered as browse.

3.2 Separation and fractionation

Separation, purification and identification of the major groups of chemical compounds were only performed on the most preferred food plants of captive Coquerel's sifakas, *A. julibrissin* and *R. copallina*. The subsequent study was then dedicated to the investigation of tannins and flavonoid glycosides within both species (Harborne, 1989; Harborne, 1999; Wollenweber 1994; Williams and Harborne, 1994). Hence, it was decided to separate crude plant extracts of either plant first, by means of a polyamide column (Markham, 1975; Fig. C.4 and Fig. C.5) and to perform additional steps of fractionation and separation on a reversed phase HPLC column until nearly pure individual plant compounds could be isolated (Tab. C.8; App. II.3). Subsequent structure elucidation should be achieved by information gained from their UV spectra, methods of mass spectrometry and NMR technique. The preparative isolation of individual plant compounds with subsequent elucidation of their chemical structure was necessary to learn more about the chemical leaf composition, the basis for subsequent quantitative determinations.

Based on the pre-trials it was decided to use leaves of *A. julibrissin* which had been harvested in May, and those of *R. copallina* which had been cut in July. While a total of 24 HPLC fractions were isolated for *A. julibrissin* (Fig. C.4), 23 HPLC fractions were isolated for *R. copallina* (Fig. C.5).

[1] The hot water extract of the leaves was a green, adhesive and mucilaginous dispersion producing threads of 1-2 cm length.

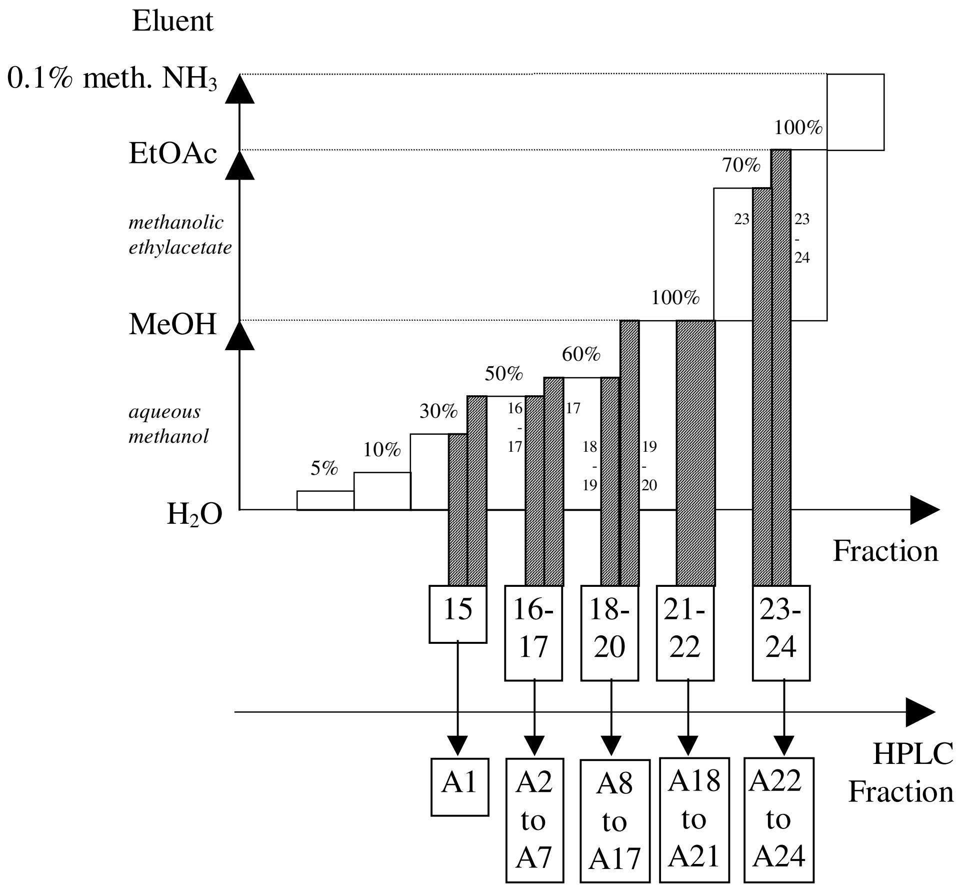


Fig. C.4: Review of separation of a crude *A. julibrissin* leaf extract (leaves harvested in May) by means of a polyamide column ("Fraction") and subsequent reversed phase HPLC column ("HPLC Fraction"). Fractions not depicted in the scheme were not further separated and discarded.

Polyamide columns exhibit a high capacity for phenolic plant compounds and have been widely used to isolate various natural products like flavonoids (Markham, 1975; Hasan *et al.*, 1996; El-Mousallamy, 1998) or hydroxycinnamic acids (Ibrahim and Barron, 1989). In case of flavonoids, all types are separated satisfactorily by polyamide columns (Markham, 1975).

The elution of phenolics from polyamide columns depends either on the ability of the solvent to replace the phenol on the hydrogen bonding site by using hydrophilic solvents, or on the ability of the solvent to form even stronger bonds with the phenol than does the polyamide (e.g. urea) (Markham, 1975). However, pre-trials on eluents best suitable for separations on polyamide columns revealed that according to the eluotropic series, starting from the polar hydrophilic end with water and increasing levels of methanol in water, turned out to be more favourable than starting from the less polar end with ethylacetate and ethylacetate-methanol mixtures. Especially the large diversity of flavonoid glycosides occurring in *A. julibrissin* leaves were stepwise and more satisfactorily separated by water and water-methanol mixtures than by starting with less polar eluents.

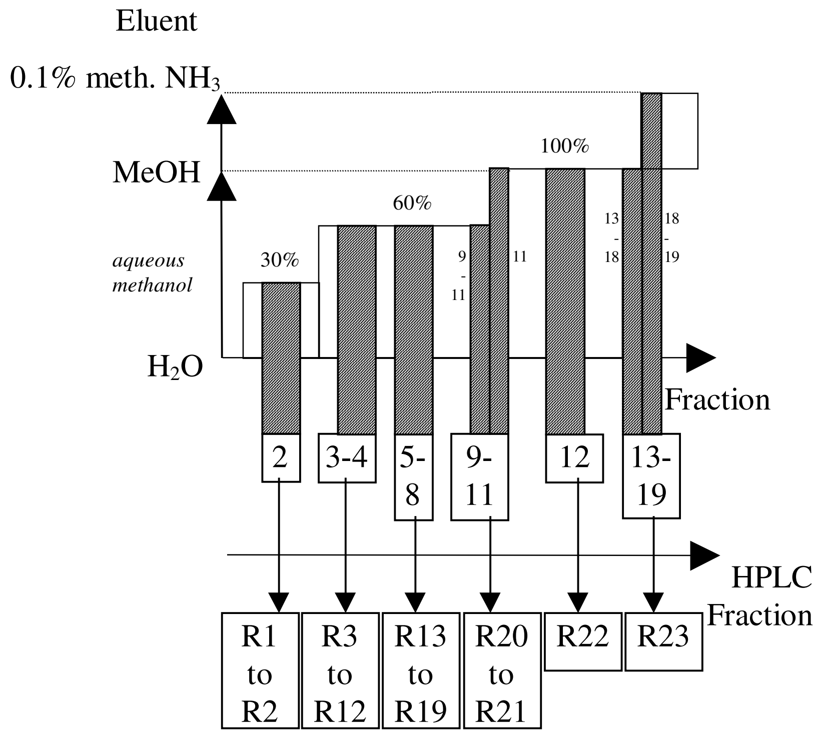


Fig. C.5: Review of separation of a crude *R. copallina* leaf extract (leaves harvested in July) by means of a polyamide column ("Fraction") and subsequent reversed phase HPLC column ("HPLC Fraction"). Fractions not depicted in the scheme were not further separated and discarded.

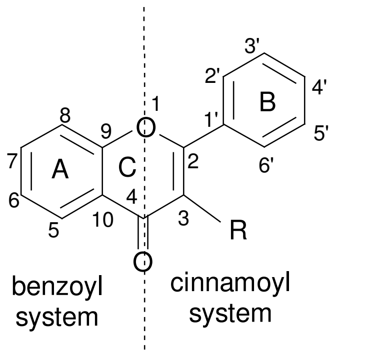
3.3 Structure identification

Elucidation of individual compounds gained by the preceding procedure revealed that most fractions of *A. julibrissin* contained flavonoids, while in *R. copallina* leaves flavonoids and monomers to dimers of hydrolysable tannins could be identified. (Higher molecular weight compounds as pentagalloylglucose could only be identified in unhydrolysed plant extracts by analytical HPLC (coinjection with the respective compound) or LC-MS; for details see chapter IV). In part, the same compounds were found to occur in both plants (Tab. C.8). Chemical structures were elucidated by their UV spectra, by mass spectrometry and NMR spectroscopy. In fractions containing several compounds, or if no NMR spectrum could be obtained due to too low amounts isolated, mass spectrometry was used to determine the molecular weight and to assess the chemical structure (details see App. II.3).

A review on the assignment of *A. julibrissin* and *R. copallina* leaf compounds to their respective HPLC chromatograms is given in Fig. C.8 and Fig. C.9.

UV Spectra
UV spectra were measured by means of a photo diode array that was linked to the analytic HPLC system (C.IV.2.1). Wavelengths between 200 nm and 600 nm were detected. As for all runs on the analytical HPLC column (reversed phase) the same gradient of aqueous phosphoric acid and acetonitril was used, the UV spectra obtained deviate in part from those of literature references in which methanol is normally used. As flavones and flavonols were the most common flavonoids found in the plants investigated, some characteristics of their UV spectra will be discussed.

The methanol spectra of flavones and flavonols generally exhibit two major absorption peaks in the region 240-400 nm. These two peaks are commonly referred to as band I (usually 300-380 nm) and band II (usually 240-280 nm) (Mabry *et al.*, 1970). While band I is mainly associated with the cinnamoyl system of the B-ring, band II is predominantly caused by the benzoyl system of the A-ring with different participation of the pyrone grouping of ring C (Fig. C.6), (Jurd, 1962; Mabry *et al.*, 1970; Markham and Mabry, 1975).



band II 240 – 285 nm

band I 300 – 400 nm

		band I
flavones	R = H	304 – 350 nm
flavonols	R = OH	352 – 385 nm
3-hydoxyl substituted flavonols	R = O-glycoside	328 – 357 nm

Fig. C.6: Range of bands I and II of flavones and flavonols in UV spectroscopy (methanol as solvent).

Increasing oxygenation of the B-ring in flavones and flavonols, like the stepwise introduction of hydroxyl groups, produces an increasing bathochromic shift in band I with each additional oxygen function. Likewise, increasing oxygenation of the A-ring in flavones and flavonols produces a notable bathochromic shift in band II, and a smaller effect on band I. Hence, as depicted in Fig. C.6, band I absorption and especially its position provides information about the type of flavonoid and its oxygenation pattern (Jurd, 1962; Mabry *et al.*, 1970; Markham and Mabry, 1975).

Although changes in the B-ring oxygenation pattern usually do not produce a shift in band II, band II may appear as either one or two peaks depending on B-ring oxidation pattern (Mabry *et al.*, 1970). So, band II of flavones and flavonols, which contain only 4'-substituent in the B-ring (e.g. kaempferol), has a single well-defined peak. For flavones and flavonols which have hydroxyl or methoxyl substituents in both the 3'- and 4'-positions (e.g. quercetin), band II shows two definite peaks or one peak and a pronounced inflection (Jurd, 1962; Mabry *et al.*, 1970). On the other hand, presence or absence of the hydroxyl group at 5 position of flavones or flavonols has a marked effect on both band I and II in the UV spectra (Mabry *et al.*, 1970).

Methylation or glycosylation of a 3-, 5- or 4'-hydroxyl group of the flavone / flavonol nucleus results in hypsochromic shifts, especially in band I (Fig. C.6). Substitution of the hydroxyl groups at positions other than 3, 5 and 4' has little or no effect on the UV spectrum (measured in methanol) (Mabry *et al.*, 1970).

Mass spectrometry

Regular fragmentation patterns in the EI mode of flavonoids, especially flavone and flavonol aglyca, are depicted in Fig. C.7 according to Mabry and Markham (1975). A common and characteristic fragmentation is the retro-Diels-Alder reaction (RDA) (Mabry and Markham, 1975; Grayer, 1989; Claeys *et al.*, 1996). Fig. C.7 shows diagnostic fragments which occur during the RDA reaction as well as during the alternative pathway. Corresponding masses of flavone and flavonol aglyca including important fragments are given in Tab. C.7. The scheme depicted in Fig. C.7 combined with the respective masses given in Tab. C.7 was very effective and helpful in evaluating the fragments found in the EI spectra of the numerous fractions and compounds investigated (see App. II.3).

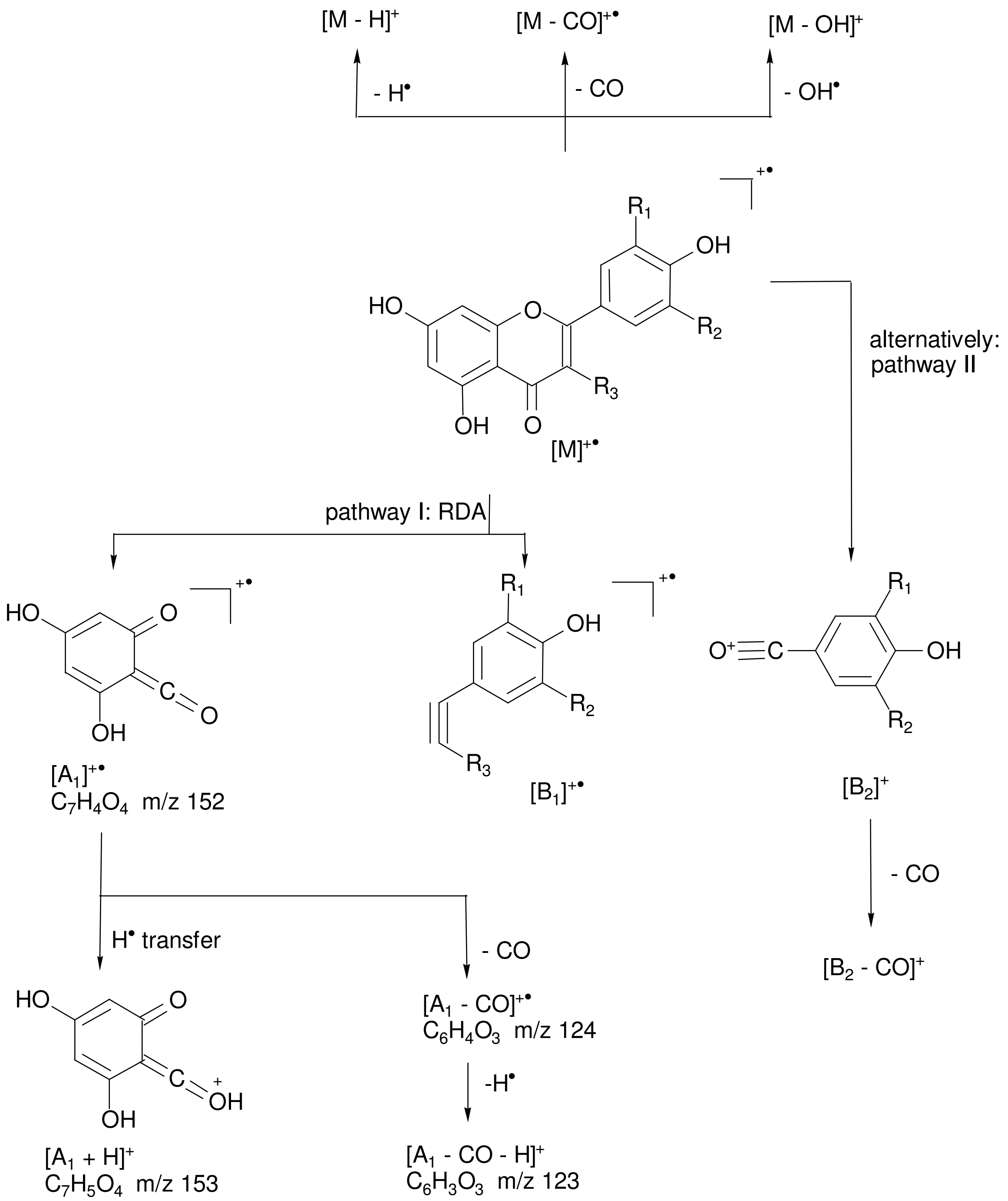


Fig. C.7: Fragmentation of flavones and / or flavonols under EI conditions. Only diagnostic fragments of retro-Diels-Alder reaction (RDA) and the alternative pathway are depicted.

Tab. C.7: Masses of fragments obtained by the EI experiment according to Fig. C.7.

substance	R_1	R_2	R_3	m/z $[M]^{+\bullet}$	$[B_1]^{+\bullet}$	$[B_2]^+$	$[B_2\text{-}CO]^+$
flavonols							
myricetin	OH	OH	OH	318 $C_{15}H_{10}O_8$	166 $C_8H_6O_4$	153 $C_7H_5O_4$	125 $C_6H_5O_3$
quercetin	OH	H	OH	302 $C_{15}H_{10}O_7$	150 $C_8H_6O_3$	137 $C_7H_5O_3$	109 $C_6H_5O_2$
kaempferol	H	H	OH	286 $C_{15}H_{10}O_6$	134 $C_8H_6O_2$	121 $C_7H_5O_2$	93 C_6H_5O
flavones							
luteolin	OH	H	H	286 $C_{15}H_{10}O_6$	134 $C_8H_6O_2$	137 $C_7H_5O_3$	109 $C_6H_5O_2$
apigenin	H	H	H	270 $C_{15}H_{10}O_5$	118 C_8H_6O	121 $C_7H_5O_2$	93 C_6H_5O

Essentially, the molecular ions of the investigated glycosides cannot be detected by EI-MS because of their high polarity. However, at increasing sample temperature they are decomposed releasing their aglyca. Thus EI-MS was used to get structural information on the aglycone of a respective glycoside. This was also true for the analyses of mixtures.

ESI-MS was employed for the identification of glycosides. However, the mass spectra obtained with this ionisation technique showed no fragments and consequently no structure-specific information. In this case collision induced dissociation (CID) and tandem mass spectrometry (MS/MS) were used for structural analyses.

The structure and elemental composition of the investigated compounds is predominantly based on the information obtained by mass spectrometric experiments. High resolution spectrometry was only performed with maesopsin-glucoside (see App. II.3, No.11).

In fraction R18 an acylated dihydrokaempferol glycoside (M_1) was diagnosed, which rearranged to apigenin under experimental conditions. For the proposed mechanism see Fig. II.7, p. 295.

A recent review on current analysis of flavonoids by means of mass spectrometric methods has been published by Cuyckens and Clays (2004).

NMR spectroscopy

To elucidate unambiguously the chemical structure of individual compounds, in particular two-dimensional experiments were utilised. Since p- and m-digallic acid methyl ester, and maesopsin-4-O-ß-D-glucopyranoside are rarely occurring compounds, HMBC experiments gave the decisive hint to structure elucidation (see also notes on maesopsin-glucoside).

Quercetin (App. II.3, No.13) turned out to be a very important and abundant flavonol aglycone in both leaf species, *R. copallina* and *A. julibrissin*, and it was investigated by HMBC and HSQC experiments. Therefore, the hydroxyl groups of quercetin could unambigously be assigned to shift values in the ^{1}H spectrum, and C2' and C5' to shift values in the ^{13}C spectrum for the first time. Quercitrin (App. II.3, No.15), the main flavonoid of *A. julibrissin* leaves, was also worthy of additional studies in two-dimensional experiments. It was also used as reference compound for further quercetin-3-O-glycosides including the novel compound. Hence, an unequivocal assignment of all carbon atoms in the ^{13}C spectrum as well as the assignment of all sugar hydroxyl protons in the ^{1}H spectrum could be achieved for quercitrin which has not been reported previously.

The novel compound A5 (App. II.3, No.17) was elucidated by means of several homonuclear and heteronuclear two-dimensional experiments and discussed in comparison to similar and known compounds. While details to all investigated compounds are presented in App. II.3, some important notes with special respect to NMR analyses are given in the following.

Notes on the isomers p- and m-digallic acid methyl ester (R16)

R16, the m- and p-isomer of digallic acid methyl ester (App. II.3, No.2), produced one peak on TLC plates, but two peaks on reversed phase HPLC. These two peaks could not be further separated by means of preparative reversed phase HPLC technique.

Exhibiting the same molecular weight, both compounds could only be identified by NMR techniques, unambigously by two-dimensional NMR spectroscopy, as p- and m-dimeric compounds consisting of a gallic acid and gallic acid methyl ester moiety, respectively. Under analytical HPLC conditions (270 nm detection wavelength) two peaks could be observed with a ratio of peak areas between 1:2 and 1:3. Based on the statistical expectation that the peak of both possible m-isomers is believed to be twice as high as the corresponding p- isomer, with the larger peak was assigned to the m-isomer and the smaller peak to the p-isomer. This agrees well with Verzele *et al.* (1983), who reported that the m-isomer constituted the main peak.

The ^{1}H- and ^{13}C-NMR spectra of both isomers in DMSO showed a ratio of 1:1. This finding seems to be in accordance with reports of Verzele *et al.* (1983) about digallic acid and of Nishizawa *et al.* (1982) about methyl digallate, showing a solvent dependent equilibrium, respectively. According to Verzele *et al.* (1983), protonating media produce two peaks on HPLC, aprotic solvents only one peak.

Whereas digallic acid was reported to be isolated after partial hydrolysis of Chinese gallotannin (from *Rhus semialata*) (Verzele *et al.*, 1983) and methyl digallate after partial methanolysis of commercial tannic acid or by synthesis (Nishizawa *et al.*, 1982), both isomeric forms of digallic acid methyl ester could be isolated from *R. copallina* leaves without any further analytical steps. It can be assumed that both isomeric forms occurred genuinely in the leaves of *R. copallina*. It could not be detected in tannic acid purchased from Fluka, neither by HPLC, nor by NMR-analysis.

Besides, the declared molecular weight for tannic acid purchased from Fluka (1701g / mol) is different from that given for Chinese gallotannin and from that given for Turkish gallotannin. Chinese gallotannin (from *Rhus semialata*) was an average molecular weight of 1434 when calculated (Nishizawa *et al.*, 1982), while Turkish gallotannin (from *Quercus infectoria*) had a calculated molecular weight of 1032 (Nishizawa *et al.*, 1983).

Notes on the maesopsin-4-O-ß-D-glucopyranoside (R2)

Maesopsin and its chemically related glycosides belong to the group of aurones, in *sensu stricto* to the group of auronols, which both belong to a minor group of flavonoids. In the chemical sense, auronols could be considered as hydrated aurones. While aurones possess a 2-benzylidenecoumaranone structure, auronols are 2-hydroxy-2-benzylcoumaranones (Bohm, 1994). Sulfuretin, that is 6,3',4'-trihydroxyaurone, has already been described to occur in the wood of the genus *Rhus* (Bohm, 1975). In this study, the auronol maesopsin-4-O-ß-D-glucopyranoside has been detected for the first time in the leaves of *R. copallina*.

On TLC plates maesopsin-glucopyranoside became visible as a bright red-orange spot with anisaldehyde / H_2SO_4. As shown on TLC plates, it was not quantitatively removed from the plant extract by casein and there was nearly no precipitation with lead acetate. According to HPLC analyses, trace amounts occurred in the leaves collected in May, with large amounts in the July leaves and lower, but substantial amounts in the leaves of October.

NMR experiments yielded conclusive data in elucidating its structure. Maesopsin-4-glucoside exists as two diastereoisomers which is due to the attachment of a glucosyl moiety to the aglycone, which itself appears as pair of enantioisomers because of the hemiketal at C2, the chiral centre of the maesopsin aglycone (for structure, numbering and analytical data see App. II.3, No.11). Therefore, a double set of signals is obtained in the ^{1}H- and ^{13}C-NMR spectra (for a discussion see Yoshikawa *et al.* (1998b) and Günther (1992)). In this study, 58 % and 42 % were found for both diastereoisomers, respectively. As far as signals have been resolved, both sets of data are depicted in the respective tables of the ^{1}H-NMR and ^{13}C-NMR experiment (App. II.3). Assignments in the ^{1}H-spectrum are based on ^{1}H and ^{1}H,^{1}H-COSY experiments. Among various two-dimensional experiments conducted, especially the HMBC experiment (App. II.3) gave the decisive hint to the position of the glucoside being attached to the aglycone.

Notes on the quercetin-3-O-α-L-rhamnopyranosyl(1→2)ß-D-xylopyranoside, the novel compound (A5)

To elucidate the structure of the novel compound A5 (App. II.3, No.17), several two-dimensional experiments were carried out, such as the homonuclear ^{1}H,^{1}H-COSY, and the heteronuclear HSQC and HMBC correlations.

Coupling constants of the anomeric protons J(C1-H/C2-H) of the sugar moieties as well as their shift values, give useful information on their mode of attachment to further sugar moieties as well as to the site (and kind) of the flavonoid aglycone. Values found in this study will be put into context with shift rules, general observations on primary and terminal sugar moieties and the attachment to the aglycone (De Bruyn *et al.*, 1979, Altona and Haasnoot, 1980; Markham and Geiger, 1994).

With respect to the question of α- or ß-linkage of the sugar moieties, it has been found that the coupling constant for J(C1-H/C2-H) for e.g. ß-D-glucose, galactose and xylose is about 7-8 Hz, while the α-D-configurations show 3-4 Hz (Markham and Geiger, 1994). Altona and Haasnoot (1980) reported 7.9 Hz for the anomeric proton of the ß-D-xylose and 3.3 Hz for the α-anomer. The anomeric proton of the xylose moiety of compound A5 couples with 7.4 Hz, which agrees well with the reported ß-configuration.

On the contrary, for ß-L-rhamnose moieties a coupling constant J(C1-H/C2-H) of 1.1 Hz was described, while for the α-linked rhamnose 1.8 Hz were given (Altona and Haasnoot, 1980). The ^{1}H-spectrum of compound A5 showed 1.54 Hz, which speaks more for the α-linked rhamnose than for the ß-anomer. Thus, the α-linkage is proposed for the rhamnose moiety in compound A5.

Chemical shift data relating to the glycosidic H-1 signal can also be of diagnostic value. The H-1 signal of a sugar attached to another sugar (referred to as "terminal") can usually be distinguished from that of a sugar directly attached to a flavonoid (i.e. "primary") in that it normally resonates upfield relative to the latter (Markham and Geiger, 1994). Exceptions are C-glycosides (C-glycosides commonly exhibit higher-field anomeric proton signals than do O-glycosides), or when the terminal sugar is apiofuranoside. Moreover, 3- and 7-O-glycosides of flavonoids, other than anthocyanidins, involving the same sugars can generally be distinguished: e.g. sugar anomeric protons of 3-O-glucosides resonate at 5.35-5.56 ppm, while anomeric protons of 3-O-rhamnosides resonate at 4.96-5.36 ppm. Furthermore, regarding terminal rhamnosyl moieties, the rhamnosyl-(1→6)-glucosides and -galactosides can be distinguished from the respective rhamnosyl-(1→2) linked residues by the chemical shift of the anomeric rhamnose proton (H-1), in that the latter resonate downfield. The presence of glycosylation or acylation on the C-2 hydroxyl of a primary sugar in both O- and C-glycosides is generally evidenced by a 0.1-0.4 ppm downfield shift in the H-1 signal of the primary sugar (Markham and Geiger, 1994).

Hence, the terminal rhamnosyl residue of compound A5 with C1-H resonating at 5.08 ppm agrees well with the shift range of 4.90-5.10 ppm reported for the anomeric proton of 2-O-α-L-rhamnosyl residues in DMSO-d_6 (Markham and Geiger, 1994). In contrast, terminal 6-O-α-L-rhamnosyl residues resonate at 4.37-4.39 ppm. It has to be further noted, that the C-methyl signal in rhamnoses varies with the site of glycosylation. In 3-O-α-L-rhamnosides the methyl signal appears in the range of 0.72-0.86 ppm, while in terminally bound rhamnoses the methyl signal occurs in the 0.90-0.93 ppm range. The latter range agrees with the 0.88 ppm found for the C6-H_3 signal of the terminal rhamnosyl residue of compound A5. Finally, the reported shift value of 5.37 ppm for the anomeric proton of the 3-O-ß-D-xyloside (as primary sugar) plus the downfield shift mentioned for glycosylation at the C-2 hydroxyl group (0.1-0.4 ppm) agrees with the 5.51 ppm experimentally found for the primary xylose moiety in compound A5. This in turn is in accordance with the resonance given for sugar anomeric protons of 3-O-glucosides: 5.35-5.56 ppm. Hence, from coupling constants and shift values found for the anomeric saccharide protons the presence of a 3-O-α-L-rhamnopyranosyl(1→2)ß-D-xylopyranoside disaccharide moiety can be deduced.

As to the aglycone, according to Markham and Geiger (1994), the A-ring protons in flavones are little influenced by the substitution pattern of the B-ring. With the usual phloroglucinol-type A-ring the substitution of H-3 in flavonols by OH has by itself almost no effect upon the signals of H-6 and H-8, provided the B-ring is unsubstituted at C2' and C6' position. Thus, with the common 5,7-dihydroxy substitution, the shift ranges are 6.16-6.25 ppm for H-6 and 6.39-6.56 ppm for H-8 in flavones and flavonols. Glycosylation of the 3-OH in flavonols with monosaccharides other than rhamnose has little influence on the B-ring resonances. A rhamnose directly linked to the 3-position either as the sole sugar or as part of a glycosidorhamnose invariably causes an upfield shift of the C2'-H and C6'-H signals by about 0.2-0.25 ppm.

Hence, comparison of C6-H and C8-H shift values of the unsubstituted quercetin aglycone (App. II.3, No.13) agree well with the aglycone of compound A5, as these protons remain largely unaffected by the saccharide moieties attached to C3-OH of the flavonol skeleton. Most affected by glycosylation of C3-OH in compound A5 is the C2'-H, while the other B-ring protons experience only little shift changes compared to sole quercetin. So, from ^{1}H-NMR data of A5, this compound is concluded to be quercetin-3-O-α-L-rhamnopyranosyl(1→2)ß-D-xylopyranoside. (For further shift values of the unsubstituted and glycosylated quercetin see App. II.3: quercitrin (No.15), hyperoside (No.14), and quercetin-3-O-α-L-rhamnopyranosyl(1→2)ß-D-galactopyranoside (No.16)).

The assignment of the carbon atoms of the novel compound was obtained by comparison with compounds containing similar sugar residues without further two-dimensional experiments (for details see App. II.3).

3.4 Compounds identified

Tab. C.8 represents all compounds which could either be conclusively elucidated, or for which constituents are proposed by means of UV spectroscopy and mass spectrometry. From the 24 fractions isolated from *A. julibrissin* leaves and the 23 fractions isolated from *R. copallina* leaves, 18 compounds could be conclusively and unambiguously elucidated by means of UV spectroscopy associated with HPLC analyses, mass spectrometry and NMR technique including various two-dimensional experiments. For the other 21 compounds, the chemical formula and the molecular weight could be calculated by means of various mass spectrometric experiments and their chemical constituents were proposed (Tab. C.8). For most additional compounds contained within the same fractions isolated (minor compounds, impurities) molecular weights could also be determined (not depicted in Tab. C.8). A detailed description of all compounds including their impurities and additionally details on these compounds that were only characterised by their UV spectra is shown in App. II.3.

Tab. C.8: Identified compounds in the leaves of *R. copallina* and *A. julibrissin*: Structures in bold are conclusively elucidated. For other compounds only constituents are proposed. Fractions from which the respective compounds have been isolated are recorded for either plant. The numbering of compounds refers to App. II.3.

No.	**identified compounds**	***R. copallina***	***A. julibrissin***
	flavonoids: flavones, flavonols and auronols		
28	dihydrokaempferol + 1 hexose moiety + 1 methoxy gallic acid moiety	R18	
7	**kaempferol**		A17, A24
8	**kaempferol-3-O-α-L-rhamnopyranoside (syn.: afzelin)**	R12	A14
9	**kaempferol-3-O-α-L-rhamnopyranosyl (1→2)ß-D-galactopyranoside**		A4
21	kaempferol + 2 deoxyhexose moieties		A7
22	kaempferol + 1 pentose moiety (isomer of R19)		A13, A21
29	kaempferol + 1 pentose moiety (isomer of A13/A21)	R19	
28	kaempferol + 1 hexose moiety	R18	
28	kaempferol + 1 hexose moiety + 1 gallic acid moiety	R18	
10	**luteolin-7-O-ß-D-glucopyranoside**	R10, R11	
11	**maesopsin-4-O-ß-D-glucopyranoside**	R2, (R8)	
24	aglycone (auronol) + 1 gallic acid moiety + 1 hexose moiety (isomer of R9)	R7	
25	auronol + 1 gallic acid moiety + 1 hexose moiety (isomer of R7)	R9	
13	**quercetin**		A15, A16, A23, R18
14	**quercetin-3-O-ß-D-galactopyranoside (syn.: hyperoside)**		A11, A19
15	**quercetin-3-O-α-L-rhamnopyranoside (syn.: quercitrin)**	R 11	A12
16	**quercetin-3-O-α-L-rhamnopyranosyl(1→2)ß-D-galactopyranoside**		A2
17	**quercetin-3-O-α-L-rhamnopyranosyl(1→2)ß-D-xylopyranoside**, novel substance		A5
18	quercetin + 1 hexose + 2 deoxyhexose (rhamnose) moieties		A1
19	quercetin + 2 hexose + 1 deoxyhexose (rhamnose) moieties		A3
20	quercetin glycoside		A6
21	quercetin + 1 gallic acid moiety		A7
22	quercetin + 2 deoxyhexose moieties		A13
22	quercetin + 1 deoxyhexose moiety (constitutional isomer of quercitrin)		A13, A21

No.	identified compounds	*R. copallina*	*A. julibrissin*
23	quercetin + 1 pentose moiety		A20
28	quercetin + 1 hexose moiety	R18	
27	quercetin + 1 hexose moiety + 1 gallic acid moiety	R17	
	gallic acid and derivatives[a]		
2	**p- and m-digallic acid methyl ester**	R16	
3	**ellagic acid**	R21	
4	**gallic acid**	R3	A8
5	**gallic acid methyl ester**	R1, R6, R15, R20, R22, R23	
24	2 gallic acid moieties + 1 hexose moiety, presumably digalloylglucose	R7	
26	gallic acid derivatives (two isomeric compounds)	R13/R14	
	other phenolics		
1	**p-coumaric acid**		A18, A22
6	**p-hydroxy benzoic acid**		A10
7	hydroxycinnamic acid derivative		A24
12	**protocatechuic acid**	R4, R5	A9

[a] For compounds determined by semi-quantitative LC-MS analyses see C.IV.3.2.

Free flavonoid aglyca and free organic acids are supposed to be degradation products through storage and / or isolation. Deoxyhexose is supposed to be rhamnose.

Most compounds listed in Tab. C.8 have not been previously shown to occur in the leaves of *R. copallina* and / or *A. julibrissin*. One compound can be classified as exceptionally novel: quercetin-3-O-α-L-rhamnopyranosyl(1→2)ß-D-xylopyranoside, which could be isolated from *A. julibrissin* leaves (fraction A5).

According to the literature on leaf composition in the genus *Rhus*, myricetin, quercetin and kaempferol are mentioned to occur as flavonol aglyca, and the flavone aglyca apigenin and luteolin are mentioned to occur periodically throughout the Anacardiaceae (see App. II.2). So, it is not surprising that flavonoids with kaempferol, quercetin, apigenin and luteolin as aglyca were found in *R. copallina* leaves in this study. In this context it should be noted, that the aglyca isolated in this study are likely to be an outcome of degradation products and do not occur genuinely in the free state. However, the exact elucidation and description of the corresponding glycosides in this study is new. The occurrence of numerous glycosides acylated with gallic acid seems to be an interesting feature of *R. copallina* leaf flavonoids and might be of interest for possible pharmacological effects. The finding of auronols and the elucidation of maesopsin-glucoside was not expected for *R. copallina* leaves based on the literature, although aurones and auronols are mentioned to occur in the Anacardiaceae (see App. II.2). Furthermore, the detection of a dihydrokaempferol glycoside acylated with methoxy gallic acid (fraction R18; No. 28) deserves some appreciation. This compound was identified by mass spectrometry yielding apigenin under ESI conditions (see Fig. II.7, p. 295). A flavanonol was not expected to occur in the leaves (compare App. II.2).

As to the flavonoids of *A. julibrissin*, only quercitrin, hyperoside and afzelin could be expected to occur in the leaves as they were mentioned in the literature, partly without giving the exact plant part of origin (Jang *et al.*, 2002; App. II.2). However, in this study numerous glycosides with kaempferol and quercetin as aglyca could be identified. Again, their detailed description and elucidation can be claimed as being new for *A. julibrissin* leaves. The occurrence of quercetin acylated with gallic acid is a remarkable and interesting finding. Furthermore, one quercetin glycoside, the quercetin-3-O-α-L-rhamnopyranosyl(1→2)ß-D-xylopyranoside, which was found in this study, can be classified as novel as it has not been described before.

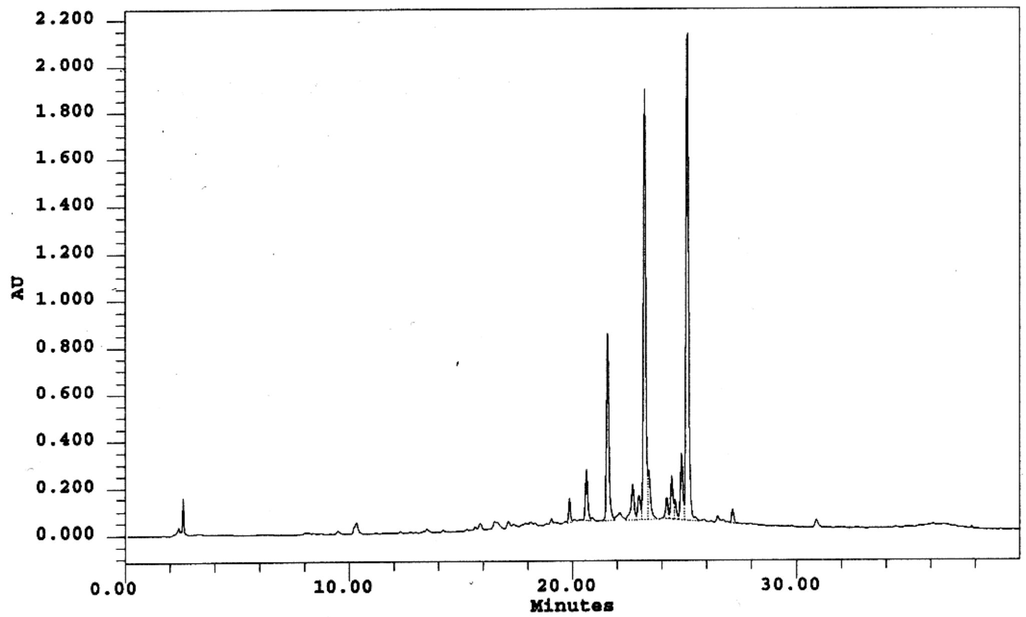
As any *Rhus* species can be used as source of commercial tannic acid (U.S. Pharmacopoeia, App. II.2), and hydrolysable tannins characterise leaves, barks and galls of the genus *Rhus* and explicitly leaves of *R. copallina* (Doorenbos and Box, 1976; Doorenbos, 1976), a rich occurrence of gallotannins and associated compounds would be expected. Although through the isolation procedure of gallotannins there is always the risk of their degradation to monomers, gallic acid and even more gallic acid methyl ester obviously occur genuinely to different extent in *R. copallina* leaves (compare Fig. C.9). It should be noted, that gallic acid and its methyl ester can be regarded as precursors for gallotannins (Kolodziej and Kayser, 1998). Similarly, the occurrence of ellagitannins is not explicitly mentioned for *R. copallina* leaves but is described to occur in the Anacadiaceae family. However, ellagic acid as artefact from a hexahydroxydiphenoyl residue could be isolated. On the other hand, the isolation of p- and m-digallic acid methyl ester could not be expected. While digallic acid was reported to be isolated by partial hydrolysis of Chinese gallotannin (from *Rhus semialata*) (Verzele *et al.*, 1983) and methyl digallate after partial methanolysis of commercial tannic acid or by synthesis (Nishizawa *et al.*, 1982), both isomeric forms of digallic acid methyl ester could be gained from *R. copallina* leaves without any further analytical steps. As depicted in Fig. C.9, both isomeric forms occur in the leaves of *R. copallina*.

Although tannins occur in barks of different *Albizia* species (Tab. II.2, p. 210), no tannins are described in the literature to occur in *A. julibrissin* leaves, and this negative result could be confirmed in this study. So, the unexpected finding of gallic acid in *A. julibrissin* leaves is more likely to originate from e.g. the degradation of acylated flavonoid glycosides than from real tannins. However, the presence of gallic acid in *A. julibrissin* leaves can be considered as new finding.

The “other” phenolic compounds found in this study (Tab. C.8) are relatively ubiquitous and their occurrence has either been previously known or could be expected in part. For instance, esters of p-coumaric acid have already been described to occur in *A. julibrissin* leaves, so that the isolation of the free acid could be expected as degradation product through isolation. On the other hand, p-hydroxy benzoic acid and protocatechuic acid have not been explicitly mentioned to occur in *A. julibrissin* leaves and are hence new findings.
For *R. copallina* leaves, the occurrence of protocatechuic acid is not explicitly mentioned in the literature, yet its occurrence is often associated with tannins and has been reported in other Anacardiaceae species (e.g. *Mangifera indica*, see App. II.2). Therefore, the presence of this compound is not a surprise.

Review on assignments of elucidated leaf compounds of *A. julibrissin* and *R. copallina* to HPLC chromatograms

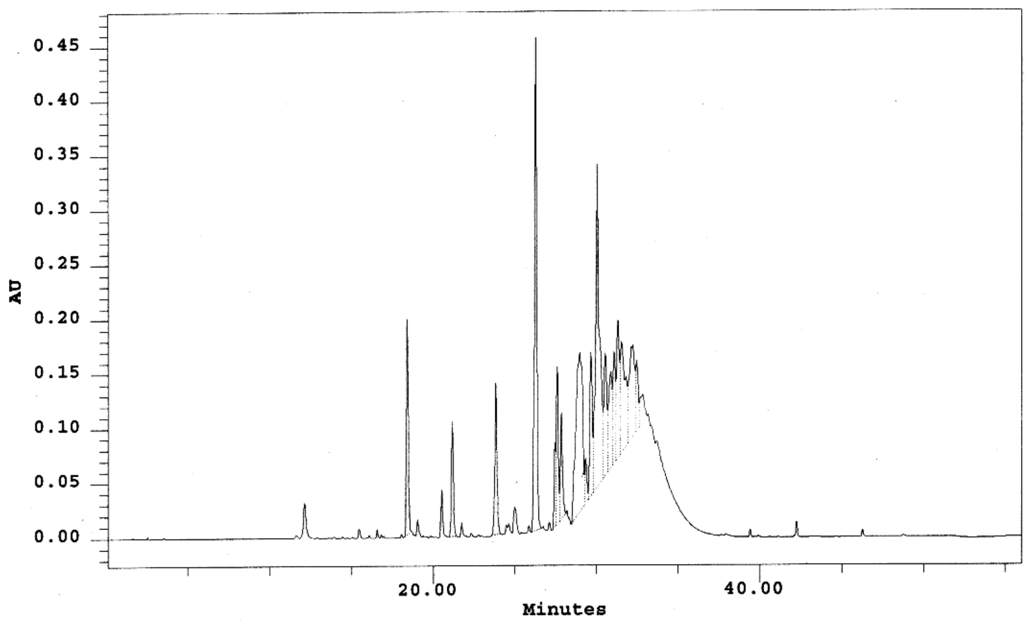
In the following, the HPLC chromatograms of *A. julibrissin* and *R. copallina* leaf extracts are shown, to which the isolated and elucidated compounds could be assigned (Fig. C.8 and Fig. C.9). For these reviews May leaves of *A. julibrissin* and July leaves of *R. copallina* were chosen. To review seasonal changes including additional compounds that were characterised by further methods, the respective HPLC chromatograms are compared in chapter C.IV (Fig. C.10, p. 122 and C.11, p. 123). The HPLC chromatograms of the other food plants (*C. canadensis*, *R. pseudoacacia*, *A. rubrum*, and *L. styraciflua*) with respect to seasonal changes are depicted in App. II.4.



ret. time (min)	name
10.3	gallic acid (A8)
19.9	p-coumaric acid derivative (free acid in A18)
20.6	quercetin derivative (A1)
21.6	quercetin-rhamnopyranosyl-galactopyranoside (A2)
22.7	kaempferol-rhamnopyranosyl-galactopyranoside (A4)
23.2	hyperoside (A11)
23.4	quercetin derivative[a]
24.2	quercetin derivative[a]
24.5	kaempferol derivative (in A6)
24.6	quercetin derivative[a]
24.9	quercetin derivative[a]
25.1	quercitrin (A12)
27.2	afzelin (A14)

[a] not isolated or elucidated due to low amounts

Fig. C.8: HPLC chromatogram of the *A. julibrissin* leaf extract (leaves harvested in May): modus: maxplot, UV detection between 210 – 450 nm.



ret. time (min)	name
12.2	gallic acid (R3)
15.6	protocatechuic acid (R4, R5)
18.4	gallic acid methyl ester (R1, R6, R15, R20, R22, R23)
21.2	auronol: maesopsin-4-O-ß-D-glucopyranoside (R2)
23.8	p-digallic acid methyl ester (R16)
26.3	m-digallic acid methyl ester (R16)

Fig. C.9: HPLC chromatogram of the *R. copallina* leaf extract (leaves harvested in July; detection at 270 nm).

C.IV Quantification of flavonoids and tannins in the food plants of *P. v. coquereli*

1 Introduction

The following chapter is dedicated to the quantification of the most common secondary plant compounds occurring in the food plants, i.e. flavonoids and hydrolysable tannins.

During previous preparative isolations and subsequent structure elucidation (chapter C.III) gallotannins have been characterised as the leading compounds of *Rhus copallina* leaves and flavonoids as main secondary leaf compounds of *Albizia julibrissin*. While to lesser extent flavonoids were also occurring in *R. copallina* leaves, no tannins could be found to occur in the leaves of *A. julibrissin*.

For a definitive evaluation of the importance of flavonoids and tannins in the diet of the sifakas, all leaf species ingested by the animals were screened for these aforementioned secondary compound groups (Tab. C.1, p. 57).

Hence, all plant specimens collected during the observation periods in both the spring and autumn were investigated for their occurrence of flavonoids and hydrolysable tannins by means of HPLC analyses. Quantification was initiated with the unhydrolysed plant extracts (Tab. C.13), which was completed by different methods of hydrolyses prior to HPLC runs (Tab. C.16 and Tab. C.17). LC-MS method was chosen to screen especially for mono- to pentagalloylglucose in *R. copallina* leaves. For this purpose the unhydrolysed leaf extracts of the different harvests were used (Tab. C.15). A gravimetric method was additionally applied to determine *R. copallina* leaf tannins (Tab. C.18).

2 Materials and Methods

2.1 Quantitative HPLC analyses

Analytical HPLC separations were performed as reversed phase chromatography by means of a RP Nucleosil® C_{18} cartridge (Macherey-Nagel; 5 µm, 250 x 4mm i.d.), Millipore Waters System, Eschborn; associated with a Waters ™ 616 Pump and Waters ™ 600 S Controller. Injections were performed by a Waters ™ 717 Autosampler, detection was carried out by a Waters ™ 996 Photodiode Array Detector. The software used was Millennium v 2.00 for data acquisition and Millennium PDA Review V.2.15 © 1994 for post processing.

(Two HPLC apparatus were used with identical equipment.)

Preparation of plant extracts

Plant material (leaf species see Tab. C.1, p. 57) was first ground and sieved according to C.I.2.2. Subsequently, all leaf material was additionally ground by means of a “Bit Beater” to be investigated by analytical HPLC runs with photodiode array detection.

Approximately 20 mg of the dried and powdered plant material (prepared by means of a mill, mortar and pistil, and sieve No. IV, DAB 7) were weighed exactly into special tubes to be extracted with 1ml 80 % (v/v) aqueous methanol by means of a Bit Beater (Biospec® Products). Tiny zircon balls were added before the tubes had been locked with a screw thread lid and provided a finely dispersed plant-solvent suspension during the shaking process at high speed.

Leaves collected in May and July were shaken for 25 seconds. When this was not sufficient for tougher leaves of some lemur food species collected during the autumn, the time in the Bit Beater was subsequently increased for all samples up to 1 minute, respectively. All suspensions were centrifuged at 14 000 rpm. for 5 minutes at –6 °C. The supernatants were deposited in Eppendorf® tubes and aliquots of these solutions were taken for qualitative and quantitative examinations by HPLC analyses. At least duplicate analyses were performed.

Comment on the extract preparation by means of a Bit Beater:
This method offers several advantages. The procedure yields a fine suspension of smashed plant cells in the appropriate extracting solvent at a given concentration. It is especially useful for small amounts of plant material without subsequent processes such as filtering, rinsing, cleaning or evaporating, which may risk the loss of material or may alter the solution’s concentration. The resulting plant compounds that are sensitive to air and light are not exposed to the harmful effects of oxygen or daylight. The zircon balls are then easily regained. As a “one step procedure”, this type of plant extraction saves time, is effective, can be easily standardised, and is useful for a wide range of analyses.

Errors can only result from the weighing method or when adding the volume of extracting medium. Hence, the Bit Beater was used to prepare plant extracts for qualitative and quantitative HPLC analyses because of its advantages for serial examinations when utilising small amounts of plant material.

Solvent system, flow rate and gradient

Solvent A: 1.5 % (v/v) aqueous phosphoric acid;
Solvent B: acetonitril (100 %).

The following gradient, at a flow rate of 1 ml / min., was used:
start with 100 % A; 0-30 min. linear gradient to 60 % A; 30-40 min. linear gradient to 20 % A; 40-45 min. 20 % A; 45-46 min. linear gradient to 100 % A; 46-56 min. 100 % A.
Every run lasted 56 min. To equilibrate the cartridge the first sample was measured twice.

All solvents used for HPLC analyses were of “p.a. grade” or “HPLC quality”.

Detection

For gallic acid and related compounds such as gallotannins, a wavelength of 270 nm was chosen, while for flavonoid glycosides and their aglyca, 350 nm was used. The maximal plot of each HPLC run was screened between 220 and 450 nm and between 210 and 450 nm for additional investigations. UV spectra of all peaks detected were recorded between 210 and 450 nm, and between 200 and 600 nm for additional investigations, respectively.

Standard solutions, calibration and formulae to calculate the contents

The following standard solutions were used to calibrate the HPLC-apparatus:
Quercitrin was used as standard to calculate the contents of quercitrin itself, flavonol and flavone glycosides and the total content of flavonoid glycosides. The standard for flavonoid aglyca was composed of myricetin, quercetin and kaempferol, which were dissolved together as one standard solution.

Gallic acid was taken to quantify the content of gallic acid itself, gallic acid-like compounds including ellagic acid, and the total content of gallotannins.

Approximately 15 mg of each standard substance were weighed exactly and dissolved in 100 ml of 100 % methanol. All standard solutions were measured as such and diluted with pure methanol to 4:1, 3:2, 1:1, 2:3 and 1:4. Injection volumes were 20 µl, respectively. At least two measurements were performed with each concentration.

As already mentioned, a wavelength of 350 nm was chosen to detect the three flavonoid aglyca, quercitrin and the total of flavonoids. Gallic acid, related compounds and the total of tannins were determined at 270 nm.

Tab. C.9: Following regression lines were found to calculate the contents of the respective compounds.

substance	regression line	standard deviation
quercitrin	AUC = (1,8736 e / µg + 0,0313) • 10^6	± 3 %
myricetin	AUC = (2,5788 e / µg - 0,0014) • 10^6	± 4 %
quercetin	AUC = (2,8353 e / µg - 0,0017) • 10^6	± 4 %
kaempferol	AUC = (3,2466 e / µg - 0,0252) • 10^6	± 4 %
gallic acid	AUC = (3,1577 e / µg - 0,0506) • 10^6	± 5 %

AUC area under the curve in the HPLC chromatogram, which is directly proportional to the content of the respective compound; the value is given in 10^6.

e injected weight of pure substance; the value is given in µg.

The point for zero concentration was included into the regression lines.

Content

The content c of secondary plant compounds investigated are referred to dry weight of the respective plant specimen and given in weight %:

$$c = \frac{f(AUC_{exp})}{e_{exp}}$$

f inverse function of regression line

AUC_{exp} area under the curve measured (experimentally determined AUC)

e_{exp} injected weight of plant specimen (dry weight)

Rate of return

$$r_{\text{rate of return}} = \frac{AUC_{exp}}{AUC_{calc}}$$

AUC_{exp} gives the (experimental) value of the corresponding peak area taken from the chromatogram.

AUC_{calc} peak area calculated using the corresponding regression line and the injected amount of the standard solution.

2.2 Quantification of unhydrolysed flavonoids and tannins

Aqueous-methanolic plant extracts were prepared by means of a Bit Beater of each plant specimen collected at the Duke Lemur Center (C.IV.2.1). Aliquots were taken and depending on plant specimen 5 or 10 µl were injected into the HPLC apparatus for analytical purposes. If necessary, further dilutions were prepared.

Within the same HPLC run, the total amount of flavonoids and tannins as well as the content of some individual compounds of either class could be quantified. All flavonoid compounds were detected at 350 nm and referred to quercitrin. The gallotannins including their monomers and dimers were detected at 270 nm and referred to gallic acid. Assignments of the UV spectra to classes of chemical compounds and individual compounds were based on data gained through previous structure elucidation and, in respect to the class of flavonoids, corroborated by UV spectra given by Mabry *et al.* (1970). Compounds not identified as belonging to either class of compounds were not included.

Contents were calculated by means of the “area under the curve” (AUC) of each peak integrated in the HPLC chromatogram, referred to the amount of the air-dried leaf specimen. (For regression lines used see Tab. C.9).

2.3 Quantification of hydrolysed flavonoids and tannins

Flavonoids

The following methods of methanolysis were developed to quantify the major flavonoid glycosides as their corresponding aglyca myricetin, quercetin and kaempferol. Reference compounds were commercial myricetin, quercetin and kaempferol, respectively. Both methods were based on the method given by Jerga (1989) using a mixture of methanol and 2 N hydrochloric acid in a ratio of 1:1.

Method A

Approximately 20 mg of the dried and powdered plant material were weighed exactly, 1 ml 80 % (v/v) methanol was added and the suspension was smashed by means of the Bit Beater for 25 seconds (C.IV.2.1). The suspension was centrifuged and 500 µl of the supernatant solution were hydrolysed in a mixture of 2 ml methanol and 2.5 ml 2 N HCl in Sovirell® glasses with Teflon® coated thread screw lids for 3 hours at 100 °C in a drying stove.

After hydrolysis the mixture was left to cool down and poured into 25-50 ml glass flasks. The Sovirell® glasses were rinsed twice with 10 % aqueous methanol and once with some pure methanol which was added to the hydrolysed solution. The solution was reduced *in vacuo* to 1-2 ml to evaporate largely the methanol (Rotavapor®, water bath of 30-40 °C) and partitioned 4 times between 10 ml of ethylacetate, respectively. The unified ethylacetate layers were washed with water in a separatory funnel (3 times with about 10 ml of water) and evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml of pure methanol and stored in Eppendorf® tubes. Right before HPLC analyses all samples were centrifuged (14000 rpm), 100 µl of each sample were transferred into HPLC test tubes and generally 50 µl, in part also 20 µl and 10 µl were injected into the HPLC apparatus.

Two samples of each plant specimen were analysed, respectively.

For the flavonoid aglyca myricetin, quercetin and kaempferol, and gallic acid, the following rate of return was found, respectively (Tab. C.10).

Tab. C.10: Rate of return of the standard compounds used.

substance	rate of return
myricetin	93 % ± 3 %
quercetin	98 % ± 4 %
kaempferol	100 % ± 2 %
gallic acid	61 % ± 11 %

Method B

Similar to method A, approximately 20 mg of the dried and powdered plant material were weighed exactly, 1 ml 80 % (v/v) methanol was added and the suspension was smashed by means of the Bit Beater. The suspension was centrifuged and 500 µl of the supernatant solution were hydrolysed in a mixture of 500 µl pure methanol and 1 ml 2 N HCl for 3 hours at 100 °C in appropriate Eppendorf® tubes with screw lids. The hydrolysed solution was poured into glass flasks and the Eppendorf® tubes were rinsed 3 to 4 times with some pure methanol, which was added to the hydrolysed solution and evaporated to dryness *in vacuo* (Rotavapor®, water bath of 30-40 °C). To remove any residual moisture, extracts were stored in an oven for 10 min. at 100 °C and, if necessary, evaporated again. The residue was dissolved in 1 ml of 100 % methanol, stored in Eppendorf® test tubes and centrifuged (5 min. at 14 000 rpm). 100 µl of each sample were filled into HPLC test tubes and generally 50 µl, but in part also 10 µl and 5 µl were injected into the HPLC apparatus.

Two samples of each plant specimen were analysed, respectively.

For the aglyca myricetin, quercetin and kaempferol, the following rate of return was found, respectively (Tab. C.11).

Tab. C.11: Rate of return of the standard compounds used.

substance	rate of return
myricetin	80 % ± 0 %
quercetin	94 % ± 3 %
kaempferol	96 % ± 3 %
gallic acid	104 % ± 6 %

Gallotannins

Plant extracts were prepared according to C.IV.2.1. Aliquots of 500 µl each were taken, transferred into 2 ml plastic tubes and brought to dryness by means of a Speed Vac centrifuge. Then, 2 ml of 4 N aqueous trifluoroacetic acid (TFA) were added to be heated for 4 hours at 100 °C in an oven. The hydrolysed plant extracts were left to cool down, poured into 25-50 ml glass flasks and the plastic tubes were rinsed three to four times with some pure methanol, which was added to the extract, respectively. The solution was evaporated to dryness *in vacuo* (Rotavapor®, water bath of 45-50 °C). To remove any residual moisture extracts were stored in the oven again for 10 min. at 100 °C and evaporated afterwards. The residue was dissolved in 1 ml of 100 % methanol, stored in Eppendorf® test tubes and centrifuged (5 min. at 14 000 rpm). 100 µl of each sample were filled into HPLC test tubes and generally 50 µl, 10 µl and in part 5 µl of different dilutions (1:1; 2:8; 1:9) were injected into the HPLC apparatus. Two samples of each plant specimen were analysed, respectively.

Tab. C.12: Rate of return of the standard compounds used.

substance	rate of return
myricetin	69 % ± 13 %
quercetin	90 % ± 2 %
kaempferol	95 % ± 4 %
gallic acid	101 % ± 11 %

2.4 LC-MS analyses on *R. copallina* leaf extracts

Preparation of plant extracts

R. copallina leaves of all three harvests (May, July and October) were extracted according to C.IV.2.1. To remove chlorophyll, these plant extracts were purified by means of a LiChrolut® Merck RP_{18} cartridge to be analysed by liquid chromatography mass spectrometry.

After equilibration of the cartridge with pure methanol, 500 µl of each previously gained extract were placed onto the column and eluted with 4 ml 50 % (v/v) methanol, respectively. A 10 ml syringe fixed by means of an adapter to the LiChrolut® column was used to achieve the high pressure needed to press extracts and eluents through the column. Extracts thus gained were evaporated to dryness *in vacuo* (Rotavapor®, water bath of 30-40 °C) and dissolved in 1 ml 80 % (v/v) methanol to be analysed by LC-MS method. 20 µl of each plant extract were screened by analytical HPLC method to assure that the spectrum of gallotannins was not significantly altered by this purification step.

Comment:
The LiChrolut® RP_{18} cartridge is very efficient in separating small amounts of compounds with different hydrophilic and lipophilic properties. The main advantage of this column is its efficacy and the speed to get an extract without disturbing lipophilic compounds, e.g. chlorophyll.
For pre-trials this sort of column was used for various pre-extractions using a mixture of water, methanol and acetic acid (50:50:1) as eluent. For reactivation the cartridge was washed with pure methanol before and after its use, respectively.

LC-MS

The negative ion electrospray spectra were obtained from a
Finnigan MAT TSQ 7000 instrument (electrospray voltage 3.5 kV; heated capillary temperature 220 °C; sheath gas and auxiliary gas: nitrogen) coupled with a Micro-Tech Ultra-Plus MicroLC system equipped with a RP_{18}-column (4 µm, 1x100 mm, Ultrasep).
Following HPLC conditions were used: gradient system from H_2O : CH_3CN 85:15 (each of them containing 0.2 % CH_3COOH) to H_2O : CH_3CN 10:90 within 15 min. and then hold at H_2O : CH_3CN 10:90 for 10 min.; flow rate 70 µl min^{-1}.

2.5 Gravimetric determination of tannins in *R. copallina* leaves

Approximately 1 g of dried and powdered *R. copallina* leaves was weighed exactly and stirred at room temperature for 30 minutes in a mixture of 30 ml isobutylmethylketone and 3 ml water by means of a magnetic stirrer. This suspension was filtered and washed with 10 ml isobutylmethylketone. The filtrate was concentrated *in vacuo* to a syrup. 25 ml water were added and concentration was continued to a volume of about 20 ml to assure complete evaporation of the ketone, until no more smell of isobutylmethylketone was perceptible. This concentrate was washed in a separatory funnel five times with 25 ml dichlormethane, respectively. The aqueous layer was evaporated to dryness, *in vacuo*, in a tared flask, dried in an oven at 70 °C overnight, cooled down at room temperature in an desiccator and weighed to determine the content of the tannin residue.

tannin content [%] = weight of tannin residue / weight of the leaves x 100

Comment**:**

This method is based on the analytical procedure of Doorenbos (1976). Since it is highly toxic, chloroform was replaced by dichlormethane in the method applied.

The "Doorenbos method" was developed to facilitate extractions of the gallotannins of *Rhus copallina* and *Rhus glabra* leaves, yielding USP quality tannic acid. According to Doorenbos (1976), this method is useful in isolating tannins for commercial purposes as well as to differentiate between the growth areas of wild *Rhus* clones, and the results from this method are in agreement with those of other methods, e.g. the hide-powder assay.

Test on the purity of the tannins

Approximately 2 mg of a gravimetrically gained tannin extract of *R. copallina* leaves were weighed exactly and dissolved in 1 ml pure methanol. An aliquot of 10 µl was analysed on analytical HPLC column and determined at 270 nm.

In a second step, tannin extracts were hydrolysed by means of TFA (C.IV.2.3, "gallotannins"). To remove last solvent residues, test tubes were dried for 10 min. at 70 °C. The hydrolysed extracts were dissolved in 1 ml pure methanol and diluted 1:10 with the same solvent. 5 µl of that solution were injected into the HPLC apparatus to test for their purity.

3 Results and discussion

3.1 Quantification of phenolic glycosides

In a first step the unhydrolysed leaf extracts of all plant specimens collected were analysed by means of an HPLC with subsequent photo diode array detection of the UV spectra (C.IV.2.1). Tab. C.13 presents the levels of flavonoid glycosides and hydrolysable tannins within the various leaf species. Quercitrin was revealed to be the most common flavonol glycoside in the melange of food plants. Based on the different absorption maxima of flavonol and flavone glycosides in the UV light (Fig. C.6, p. 101), these two groups of flavonoids were recorded separately for each food plant. The "total flavonoids" refers to all flavonoids occurring in any species which were detected at 350 nm and were referred to quercitrin as reference compound. "Total tannins" designates all gallic acid and ellagic acid derivatives which could be determined at 270 nm and were referred to gallic acid.

It is obvious that *A. julibrissin* leaves show highest contents in flavonoids, which all belong to the group of flavonol glycosides. On the contrary, *R. copallina* leaves are highest in hydrolysable tannins (Tab. C.13).

Not depicted above is that apart from *A. julibrissin* leaf specimens, hyperoside was tentatively identified in the leaves of *Acer rubrum* harvested in May (0.36 %) and in *Photinia glabra* autumn leaves (0.35 %). Kaempferol-rhamnopyranoside (afzelin) was furthermore detected in *Cercis canadensis* leaves collected in spring (0.16 %) and as trace amounts in those leaves harvested in the autumn. It was tentatively identified as trace amounts in *A. rubrum* leaves taken in the spring.

Tab. C.13: Review on the major leaf compounds investigated in unhydrolysed extracts of all leaf specimens collected. Values are given in % dry weight.

plant specimen	flavonoids: flavonol glycosides: quercitrin	flavonol glycosides: total	flavone glycosides	total flavonoids	total tannins
Albizia julibrissin					
May	1.73	4.85		4.85	
May, tips	1.78	4.29		4.29	
July	1.83	2.79		2.79	
October, young leaves	1.85	3.21		3.21	
October, old leaves	2.28	2.68		2.68	
Rhus copallina					
May		0.73	trace am.	0.81	27.85
July			trace am.	0.71	28.05
October		0.57	trace am.	0.57	17.77
Liquidambar styraciflua					
May	0.57	1.43		1.91	1.11
October	0.65	1.70		1.87	0.45
Cercis canadensis					
May	1.12	2.68		3.23	0.19
October	1.03	1.85		2.10	
Robinia pseudoacacia					
May			3.03	3.03	
October			1.46	1.46	
Acer rubrum					
May		1.05		2.77	18.02[a]
October		0.65		1.48	12.28
Liriodendron tulipifera[b]		2.14		4.35	
Vitis rotundifolia[b]	0.17	0.91		0.91	1.69
Photinia glabra[b]	0.22[c]	1.14		5.15	

[a] Due to huge amounts of the main component, HPLC runs measuring different concentrations were used to calculate the total content of tannins.

[b] harvested in October

[c] tentatively identified as quercitrin.

Grey fields indicate that these values were further used to calculate corresponding amounts eaten by the animals. Plant specimens without grey coloured fields were not ingested during either observation period.
The sum of flavonol glycosides referred to quercitrin as standard gives the total of all glycosides identified as containing myricetin, quercetin or kaempferol as aglycone as well as compounds showing closely related UV spectra. As flavone glycosides only compounds based on the apigenin- or luteolin-aglycone-type including related compounds (like acacetin (methoxyapigenin) or vitexin (C-glucosyl-apigenin) etc.) were regarded.
The total of flavonoids is referred to quercitrin as standard and comprises all compounds identified as flavonoids (Mabry *et al.*, 1970). All flavonoids were measured at 350 nm, while tannins and related compounds were measured at 270 nm.

The majority of flavonols in all leaf species investigated comprised glycosides of the quercetin type aglycone, followed by kaempferol aglycone. Apart from few exceptions, flavonols built the largest group of flavonoids in all plants analysed. Only *Robinia pseudoacacia* showed high contents in flavones.

Not all samples were unambiguously identified as containing other types of flavone glycosides (e.g. 4',7-dihydroxyflavone or 3',4',7-trihydroxyflavone as aglyca) based on their UV spectra when compared to those given by Mabry *et al.* (1970). The group of flavone glycosides in this study is confined to those containing apigenin, luteolin and related aglyca.

Another interesting note (not depicted in the table) can be made on the class of aurones. Except for *R. copallina* leaves, aurones could be detected in *Liquidambar styraciflua* leaves from both seasons. In this context it is interesting to note that those compounds that are present yet deemed rare are in evidence among the selected plant species undergoing analysis.

V. rotundifolia was chosen as a plant species, that could only be eaten outside in the natural habitat enclosure, as it was not brought into the animals' cages by the staff. This species should corroborate the aspect of food selectivity in respect to groups of secondary compounds shown by the sifakas.

In Tab. C.14 the investigation on the contents of flavonoid compounds in *A. julibrissin* leaves is presented. It is shown that there is a relative overall increase in quercitrin during the year, while there is an overall decrease in hyperoside. Despite that, the absolute content of flavonoids decreases during the year.

Tab. C.14: Relative abundance of individual components and absolute content of flavonoids in leaves of *A. julibrissin* of different age referred to dry weight.

	relative abundance (%)				
flavonoid glycosides	**May**	**May, tips**	**July**	**October, young leaves**	**October, old leaves**
quercetin triglycoside (A1)	1.8	2.5			
quercetin-gal-rha	13.2	14.1	trace am.[a]		
kaempferol-gal-rha	3.4	trace am.[a]			
hyperoside	33.9	35.2	14.5	4.6	7.4
kaempferol-derivative (in A6)	3.6	trace am.[a]	3.2		
quercitrin	35.7	41.4	65.7	57.6	84.9
afzelin	trace am.[a]		9.7	6.5	trace am.[a]
total flavonoids [%] (m/m)	4.85	4.29	2.79	3.21	2.68

[a] trace amounts indicate that the UV spectrum of the respective substance was monitored but the corresponding peak was not integrated due to too low amounts. If no value is given, no UV spectrum was registered and the respective peak in the HPLC chromatogram was absent or too small for a definite assignment. Reference compound for all glycosides was quercitrin.

If correlated to feeding data, it can be stated that tips of May leaves were left over and normally dropped by the animals, although this non-selection, could not be predicted based on the assayed flavonoid levels.

For leaves harvested in July, no feeding data were recorded. Hence, contents of July leaves are informative for the overall trend of secondary compounds during the year, but are excluded from correlations to feeding data.

In autumn, *A. julibrissin* leaves were eaten less frequently than in the spring. If at all, only young leaves were ingested, while old ones were refused. However, with respect to the pattern of flavonoids, there is a huge difference between the levels in the spring and autumn leaves. It is tempting to hypothesise that the feeding behaviour is linked to the occurrence and content of individual flavonoid glycosides.

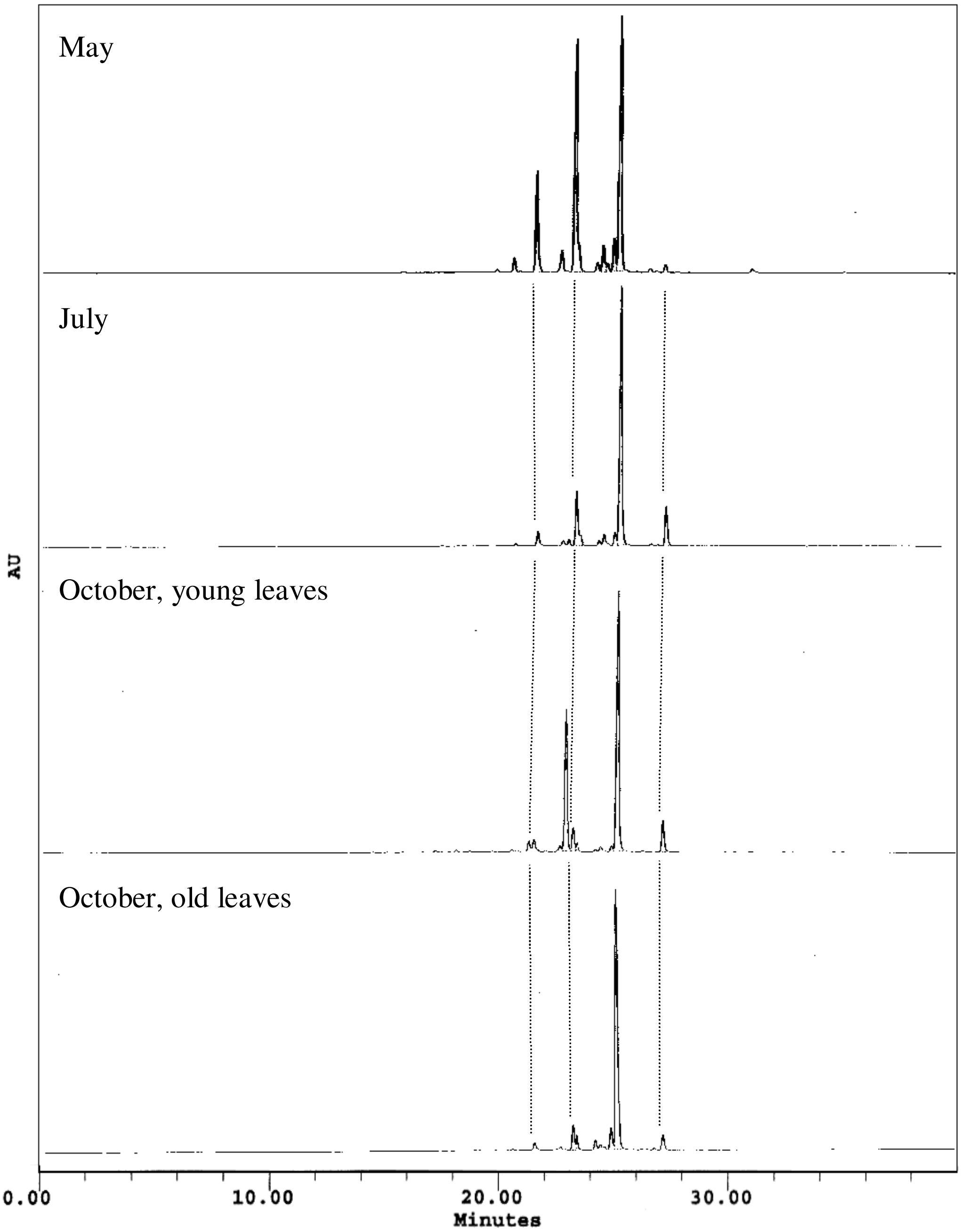
However, for dosage calculations of flavonoid glycosides ingested by the animals, leaves harvested in May and young leaves of October were used (Tab. C.25, p. 166).

Fig. C.10 presents the corresponding HPLC chromatograms to illustrate seasonal fluctuations of the flavonoid pattern in the leaves of *A. julibrissin*. Only the main peaks were assigned to compounds isolated. As the pattern of flavonoids of the leaf buds (tips) is essentially the same as shown for the developed leaves of May, the HPLC chromatogram of the tips is not depicted here. For a detailed assignment of all peaks see chapter C.III, Fig. C.8, p.110.

The tannin levels in *R. copallina* leaves, in the May and July leaves show a nearly identical content, while autumn leaves are diminished in value. However, the contents of tannins in *R. copallina* leaves is confined to the total content of the respective season (Tab. C.13) as repeated measurements showed that during storage, the portion of gallic acid is raised. Other compounds changing during storage were the contents of ellagic acid and pentagalloylglucose. While initial measurements of pentagalloylglucose could not be unambigously detected in *R. copallina* leaves of May and July, in subsequent measurements, its occurrence could be proved by coinjection with an authentic sample. In autumn leaves it could be detected during early measurements. In the HPLC chromatogram (Fig. C.11), pentagalloylglucose directly followed the para-isomer and preceded the meta-isomer of digallic acid methyl ester (contained in fraction R16), which was corroborated by the coinjection of authentic pentagalloylglucose and the isolated fraction R16. However, with respect to different influences by storage conditions it was relinquished to give detailed information on the contents of individual components as done for *A. julibrissin*.

As to ellagic acid, it could only be detected in *R. copallina* leaves of July with 0.20 %. Apart from *R. copallina* leaves, it could only be found in *A. rubrum* leaves of spring (0.19 %) and in *V. rotundifolia* leaves harvested in the autumn (0.95 %). It is reasonable to assume that the occurrence of free ellagic acid is caused by the degradation processes during storage.

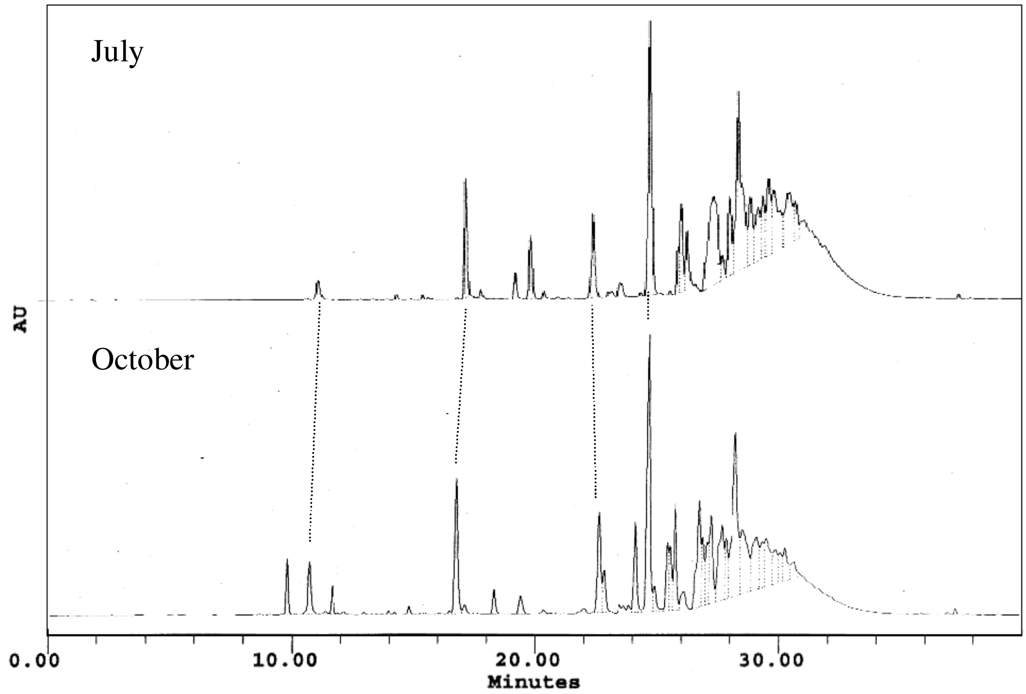
In Fig. C.11 the composition of *R. copallina* leaves harvested in July and October is depicted. For a detailed assignment of isolated compounds to the peaks detected, see also chapter C.III, Fig. C.9, p. 111.



ret. time (min)	name
20.6	quercetin triglycoside (A 1)
21.6	quercetin-rhamnopyranosyl-galactopyranoside
22.7	kaempferol-rhamnopyranosyl-galactopyranoside
22.9	flavonol glycoside[a]
23.2	hyperoside
24.5	kaempferol derivative (in A 6)
25.1	quercitrin
27.2	afzelin

[a] The flavonol glycoside with a retention time at 22.9 min. emerged first in July leaves with largest peak in young leaves of October. It could not be detected in leaves of May.

Fig. C.10: HPLC chromatograms, which show the different flavonoid composition in *A. julibrissin* leaves of May, July, and young and old leaves of October (from top to bottom, detection at 350 nm).



ret. time (min)	name
9.8	gallic acid derivative
10.7	gallic acid
16.8	gallic acid methyl ester
19.4	auronol: maesopsin-4-O-ß-D-glucopyranoside
20.3	auronol
22.6	p-digallic acid methyl ester
24.1	pentagalloylglucose
24.6	m-digallic acid methyl ester

Due to different HPLC apparatus, retention times differed slightly. Assignments of times and peaks in the table is depicted for October leaves.

Fig. C.11: HPLC chromatograms, which show the different composition of the major compounds in *Rhus copallina* leaves of July and October. (Detection at 270 nm).

Another interesting observation on the seasonal differences in tree leaves, is the occurrence of aurones (not depicted here). Only the aurones detected by UV spectra in *R. copallina* leaves from the July were integrated. Its content was reduced in autumn leaves while it was nearly absent in the leaves of May. This is in accordance with TLC-detection whereby aurones of this type yield a bright-red orange spot if detected with reagents containing sulphuric acid.

However, *R. copallina* leaves were vigorously eaten during both times of observation and correlation to the feeding behaviour will show if the amount of tannins is held constant throughout the year by the animals, or whether the amount ingested fluctuates with leaf content.

In the following, different methods of hydrolysis were performed to reveal the aglyca which the glycosides are based on. Moreover, complementary information gained by different methods corroborate the results already presented above and complete the picture of compounds vital to the study animals.

3.2 Semi-quantitative estimation of gallotannins by LC-MS

LC-MS analyses were performed on *R. copallina* leaf extracts of May, July and October to screen especially for the occurrence of mono- to pentagalloylglucose while taking into account possible seasonal differences (Tab. C.15).

Tab. C.15: *R. copallina* leaf extracts (of May, July and October leaves) were screened by LC-MS analyses. Measurements were performed in the negative ESI-mode.

compound	harvest May	harvest July	harvest October
gallic acid	n.d.	+	n.d.
gallic acid methyl ester	+++	+++	+++
protocatechuic acid	n.d.	n.d.	n.d.
monogalloylglucose	+	+	+
digalloylglucose	(+)	-	-
trigalloylglucose	-	-	-
tetragalloylglucose	+	+	(+)
pentagalloylglucose	+++	+++	+++
450 ($[M-H]^-$, m/z 449)	-	+	+
448 ($[M-H]^-$, m/z 447)	-	+	+
valonic acid	-	-	-
ellagic acid	-	-	-
hexahydroxydiphenic acid	-	-	-

+++ main component
+ present
(+) trace amounts
- not found
n.d. not detected

In accordance with previous investigations, major components in *R. copallina* leaves are gallic acid methyl ester and pentagalloylglucose. While mono- and tetra-galloylglucoses could be found in nearly all samples, di- and tri-galloylglucoses were largely lacking.

The fact that gallic acid as such, could not be detected in any case by LS-MS analyses might be due to analytical conditions. Obviously, most of the free gallic acid, which was presumed to have been generated through storage conditions, was converted into gallic acid methyl ester during LC-MS analyses, as HPLC control of prepared extracts clearly showed the occurrence of more or less free gallic acid in all three provenances.

Protocatechuic acid, which was preparatively isolated from July leaves (chapter C.III, Tab. C.8, p. 107), could not be detected by this method.

The molecular weight of 448 might be ascribed to a flavonoid component like quercitrin which could be found in July leaves through preparative isolations. However, during HPLC runs to quantify flavonoid components, quercitrin could not be detected in either leaf sample (see Tab. C.13). However, it is reasonable to assume that MW 448 originates from a flavonoid compound.

The molecular weight of 450 coincides with that of maesopsin-glucopyranoside which could be detected by HPLC analyses in the leaves of all three seasons. It remains obscure why it was not detected in leaves of May by LC-MS.

Ellagic acid and its precursor hexahydroxydiphenic acid are yielded normally from ellagitannins through methods of hydrolyses and were hence not expected to occur genuinely in the plant.

It can be summarised that LC-MS resulted in an appropriate method to screen for tannin components of higher molecular weight which are far more difficult to determine by e.g. preparative isolations with subsequent mass spectrometry. Another option to detect pentagalloylglucose was coinjection during an analytical HPLC run (see above).

3.3 Quantification of flavonoid aglyca

To analyse the leaves for their occurrence of flavonoid aglyca myricetin, quercetin and kaempferol, a methanolysis was performed on all leaf specimens (Tab. C.16). Reference compounds were commercial myricetin, quercetin and kaempferol, respectively.

Tab. C.16: Review on all plant extracts after methanolysis (method B).
Values are given in % dry weight.

plant specimen	myricetin	quercetin	kaempferol	total aglyca
Albizia julibrissin				
May		1.63	0.15	1.96
May, tips		1.42	0.08	1.60
July		1.00	0.19	1.29
October, young leaves	0.33	0.83	0.11	1.28
October, old leaves		1.02	0.09	1.16
Rhus copallina				
May		0.24	0.09	0.45
July		0.22	0.07	0.49
October		0.25	0.08	0.50
Liquidambar styraciflua				
May	0.37	0.21	trace am.	0.60
October	0.32	0.22		0.51
Cercis canadensis				
May	0.39	0.35	0.05	0.76
October	0.29	0.40	trace am.	0.66
Robinia pseudoacacia				
May				1.15
October				0.44
Acer rubrum				
May		1.14	0.11	1.29
October		0.71	0.06	0.78
Liriodendron tulipifera[a]		0.23	0.12	0.89
Vitis rotundifolia[a]				0.43
Photinia glabra[a]		0.52	trace am.	1.60

[a] harvested in October

Myricetin, quercetin and kaempferol were individually calculated, referred to its respective standard. “Total aglyca” gives the sum of all aglyca integrated referred to quercetin as common standard.

Grey fields indicate that these values were further used to calculate corresponding dosages eaten by the focal lemurs. Plant specimens without grey coloured fields were not ingested during either observation period.

First method A was developed to hydrolyse flavonoid glycosides into their corresponding aglyca myricetin, quercetin and kaempferol showing high rates of return. However, extraction with ethylacetate bears the risk of losing substantial amounts of more hydrophilic C-glycosylic compounds like vitexin, which has been criticised (in the comment of DAB 9, monograph of “Crataegi folium cum flore”). In view of the large number of plant specimens to be analysed and with respect to the problem of ethylacetate extraction, method A was replaced by method B (C.IV.2.3, “flavonoids”). Regarding contents of the aglyca myricetin, quercetin and kaempferol, both methods yielded comparable results. But as expected, method B, was preferred in the investigation of other compounds. After methanolysis by method B, anthocyanidins were found in young autumn leaves of *A. julibrissin* as well as in leaves of *Liriodendron tulipifera*, *Photinia glabra,* and respectively in *Robinia pseudoacacia*, *Cercis canadensis* and *Acer rubrum* leaves from both seasons. However, as anthocyanidins show a minimum at 350 nm, their content is not included in the contents determined for flavonoid aglyca. As pointed out by Jurd (1962), the intensity and position of the visible maximum shifts considerably with changes in pH and solvent, so it cannot be concluded from literature reference chromatograms which sort of anthocyanidins were detected in this study.

As expected, the highest contents of aglyca were found in *A. julibrissin* leaf specimens with quercetin as the main aglycone. Apart from the exceptions already mentioned, quercetin followed by kaempferol were generally the most ubiquitous aglyca in the leaf specimens investigated.

Initially, the idea to hydrolyse gallotannins together with flavonoids by means of the same hydrolysis method, the methanolysis, was subsequently discarded and deemed highly problematic.

According to methanolysis method A, the rate of return for pure gallic acid amounted to 61 ± 11 % and between 78 and 100 % of gallic acid was obtained as gallic acid methyl ester. Although omission of the ethylacetate step raised the rate of return up to 104 ± 6 %, and the portion of gallic acid methyl ester was reduced to 70 %, methanolysis in general turned out to be too soft to break down the hydrolysable tannins, especially those contained in *R. copallina* leaves. Hence, tannins were hydrolysed by means of trifluoroacetic acid (C.IV.2.3, “gallotannins”).

3.4 Quantification of tannin derived gallic acid

By means of TFA-hydrolysis (C.IV.2.3, “gallotannins”), hydrolysable tannins were largely broken down to gallic acid and, as far as occurring, to ellagic acid. Only to a minor extent could some compounds closely related to gallic acid not be further hydrolysed (identified by their UV spectra).

As already found by investigations on the unhydrolysed leaf extracts, *R. copallina* and *A. rubrum* leaves contained the largest amounts of gallic acid and total hydrolysable tannins.

Therefore, comparable results were obtained for the total content of hydrolysable tannins after TFA-hydrolysis as for the unhydrolysed leaf extracts.

The method of TFA-hydrolysis itself was validated with *R. copallina* leaves from July, commercial tannic acid, gallic acid and in addition, all three flavonoid aglyca were investigated. Results showed that aqueous TFA was superior to methanolic TFA, and 4 hours of hydrolysis were superior to two hours. Furthermore, methanol as solvent led to a range of methylation reactions, which did not occur with aqueous TFA, which in turn resulted in a reliable content of gallic acid and gallic acid methyl ester after hydrolysis. On the other hand, this method was not appropriate to hydrolyse flavonoids, as compared to methods of methanolysis (see above) only reduced contents of flavonoids were obtained.

Tab. C.17: Review on plant extracts after TFA-hydrolysis. Values are given in % dry weight.

plant specimen	gallic acid	ellagic acid	total[a]
Albizia julibrissin			
May	0.08		0.08
May, tips	0.07		0.07
July			
October, young leaves	0.04		0.04
October, old leaves	trace am.		trace am.
Rhus copallina			
May	19.77		22.86
July	17.06		20.56
October	10.79		17.35
Liquidambar styraciflua			
May	2.06	0.46	2.50
October	1.31	0.48	1.78
Cercis canadensis			
May	1.60		1.69
October	1.22		1.22
Robinia pseudoacacia			
May	trace am.		trace am.
October	trace am.		trace am.
Acer rubrum			
May	13.58	0.18	20.04[b]
October	10.69		15.73[b]
Liriodendron tulipifera[d]		0.07[c]	
Vitis rotundifolia[d]	0.70	6.95	7.63
Photinia glabra[d]			

[a] "Total" gives the total content of gallic acid, not completely hydrolysed related compounds and, as far as occurring, ellagic acid.

[b] Due to the large amount of gallic acid, HPLC chromatograms using different concentrations were used to calculate the total content of gallic acid, related compounds and ellagic acid.

[c] verified by methanolysis

[d] harvested in October

3.5 Gravimetric determination of tannins in *R. copallina* leaves

The gravimetric determination of tannins in *R. copallina* leaves is based on the method of Doorenbos (1976), a method using selective solvent extraction which has been developed to determine gallotannin levels in leaves of *R. copallina* and *R. glabra*. In this study, a slightly modified procedure has been applied (C.IV.2.5). Two determinations were performed on each leaf harvest, respectively.

Tab. C.18: Gravimetric determination of leaf tannin contents of *Rhus copallina* with respect to seasonal differences. Values given are referred to leaf dry weight.

harvest	content % (m/m)
May	28.02
July	28.03
October	21.65

In agreement with results of HPLC determinations (C.IV.2.1) of the unhydrolysed and hydrolysed leaf extracts, both the May and July leaf harvests contain nearly the same high contents of gallotannins, while October leaves exhibit reduced contents.

To test for purity of the obtained extracts, samples were examined by HPLC analysis, and in addition by various TLC systems (TLC-systems 1 and 3; anisaldehyde / H_2SO_4; $FeCl_3$-reagent). As usual, HPLC chromatograms were detected at 270 nm for tannins and at 350 nm for flavonoids.

Viewed at 270 nm, gallic acid was the main component, followed by gallic acid methylester, and to a far lesser extent other gallic acid derivatives occurred. Flavonoids detected at 350 nm occurred in negligible amounts and were hence not integrated in the HPLC chromatogram.

After additional TFA-hydrolysis (C.IV.2.3, “gallotannins”) leaf tannin extracts were hydrolysed yielding gallic acid and gallic acid methyl ester. While a portion of approximately 59 % of gallic acid and gallic acid methyl ester was calculated to occur in the tannin extract of May leaves after hydrolysis, about 67 % were calculated for the extract of July leaves and 85 % for the extract of October leaves. Differences in the portion of gallic acid and gallic acid methyl ester indicate seasonal changes in leaf tannin composition.

It can be summarised that the contents of tannins in *R. copallina* leaves and the contents of flavonoids in *A. julibrissin* leaves decreased during the year with a marked change in the flavonoid composition. From a review of the literature (chapter C.II), high contents of hydrolysable tannins could only be expected for *R. copallina* leaves. Neither the high content of hydrolysable tannins in *A. rubrum* leaves, nor the high content of flavonoids in *A. julibrissin* leaves could be expected based on the published studies. The special pattern of flavonoid levels and the contents of flavonoids and tannins that changed with seasons are important results of this investigation. Correlations of the contents of these compounds to amounts ingested by the animals will show how animals reacted to this challenge (chapter C.VI).

C.V Mucilage analyses of *Albizia julibrissin*

1 Introduction

During preliminary assays for this study (C.III.3.1), a third group of secondary plant compounds has been detected in one of the major food plants. The leaves of *Albizia julibrissin* were found to contain a mucilaginous type of compound, consisting of high molecular weight carbohydrates, that has not been characterised before.

Since mucilage containing phytotherapeutics are already used to cure different gastrointestinal disorders including diarrhoea for humans, the idea presents itself that this plant's mucilage may offer beneficial gastrointestinal properties to the lemurs as well.

Depending on the plant's site of production, function, storage and secretion, polysaccharides can be divided into structural polysaccharides (cellulose, hemicellulose and pectins), reserve polysaccharides, and mucilaginous compounds such as gums and neutral or acidic plant mucilages (Steinegger and Hänsel, 1988). While gums are produced and secreted at the site of a plant wound it will build a pathological process (called gummosis), whereby mucilaginous compounds in *sensu strictu* are (pre)stored, e.g. in vacuoles.

Chemically, most gelling and mucilaginous compounds are heteropolysaccharides of either neutral or acidic character. For instance, polyuronic acids are characteristic of acidic mucilages, pectins and gums (Steinegger and Hänsel, 1988; Rimpler, 1999). The tertiary and quaternary structure of plant polysaccharides is decisive for their water-binding and gelling capacity, which in turn determines the viscosity of different concentrated gels. The structure further determines their use in medicine, in the pharmaceutical technology and in the food industry as emulsifying, stabilising or gelling agents.

Hence, in this study the leaf mucilage of *A. julibrissin* was first characterised by its physico-chemical properties such as the "swelling number" when the plant material was soaked in water, its pH and possible interactions with other food compounds such as tannins. Tannins were simultaneously ingested by the sifakas while eating e.g. *R. copallina* leaves.

A preliminary chemical characterisation of the leaf mucilage was performed and their monosaccharide constituents, their type of linkage and some sequential information were obtained. This relied mainly on hydrolytic techniques followed by GC/MS analysis of the chemically modified monomers. In a complementary way, the mass spectrometric analysis of short oligomeric fragments of the polysaccharide were obtained by partial hydrolysis, which allowed for the deduction of general sequence patterns. This information was used for the classification of the polymer to known groups of plant polysaccharides. This characterisation of the plant polysaccharide will be useful to predict its possible usefulness in the treatment of gastrointestinal disorders.

2 Materials and Methods

2.1 Characterisation of physico-chemical parameters of *A. julibrissin* leaf mucilage

Swelling number (Quellungszahl, QZ)

The determination of the "swelling number" is a conventional method to characterise the gelling properties of mucilage from plant specimens (Ph.Eur. II; DAB 9; DAC 1986; Stahl and Schild, 1981). The "swelling number" gives the volume including the welling mucilage which clings to the ground plant tissue, and is measured by leaving it to soak in an aqueous solution for 4 hours. The value is referred to 1 g plant material (Ph.Eur. II; Stahl and Schild, 1981). As this is a conventional method, it needs to be adjusted to individual plant specimens with respect to the input of weight and the degree of fineness required (compare monographs of DAB 9, DAC 1986).

Definition of the swelling number: QZ = v/w [cm^3 / g]

Procedure: The ground and exactly weighed plant material (normally approximately 1 g) is first moistened by 1 cm^3 ethanol (96 % v/v) and left to soak in 25 cm^3 water for 4 hours in a sealable 25 cm^3 cylinder graduate by 0.5 cm^3. The cylinder is shaken or stirred every 10 minutes during the first hour. Floating particles are removed after 1.5 hours after starting the assay by turning the cylinder round its longitudinal axis. After a total of 4 hours, the volume is recorded.

In this study, different mills (Braun® or Moulinex®), a mortar with pistil, and sieve No. IV (DAB 7) were used to grind and sieve the plant specimens. 0.25 g input were used of ground and powdered *A. julibrissin* leaves, while 0.5 g were used of the other plant specimens collected at the DULC, respectively.

Normally, assays are performed in triplicates and averaged, but in this study, most plant specimens had 6 to 9 assays performed.

Reference substance
Plantaginis ovatae testa (DAC 1986; Ispaghula husks[1]) from *P. ovata* (syn. *P. ispaghula*)

Gelling properties of *A. julibrissin* leaves

For the following investigations *A. julibrissin* leaves collected in May were used.

a) Preparation of a simple gel with *A. julibrissin* leaves: powdered leaf material was stirred in water (q.s.) yielding a green gel.
b) Based on experiments from Mital and Adotey (1973), emulsions of *A. julibrissin* gel with paraffin were prepared. First, paraffin (paraffinum subliquidum; q.s.) was added to the simple *A. julibrissin* gel. Then, some tannin extract (C.IV.2.5) was dispersed in paraffin (q.s.) to be subsequently added to the *A. julibrissin* gel emulsion.
c) 4 % and 8 % (m/v) gel preparations: 4 % and 8 % (m/v) aqueous dispersions of ground *A. julibrissin* leaves and Plantaginis ovatae testa (Ispaghula husks; reference compound) were respectively left to soak in water to be centrifuged afterwards. Supernatant gels were used for experiments.

[1] Ispaghula Husk, British Pharmaceutical Codex (BCP) 1979; Psyllium Husk, United States Pharmacopoeia (USP) XXI (1985)

2.2 Isolation and chemical characterisation of *A. julibrissin* leaf mucilage

Gel filtration and fractionation

The plant mucilage of *A. julibrissin* was fractionated by gel filtration on a sepharose gel. Carbohydrates were detected by PAS and proteins by Bradford reagent, respectively. Guided by detection with PAS reagent, fractions were collected and pooled. Prior to further analyses, the pooled fractions were dialysed extensively against distilled water. Aliquots of the pooled fractions were subjected to structural analyses by means of GC, GC-MS and ESI-MS/MS experiments.

Fractionation of the plant mucilage was performed at the laboratory of Dr. M.-L. Enss (MHH).

Preparation of the plant material

50 mg of the dried and powdered leaves of *A. julibrissin* (harvested in May) were left to soak in 10 ml water for 24 hours. The dispersion was centrifuged and sieved (PM 30) to gain a gel with a molecular weight of at least 30 000 Dalton. 4 ml of this solution were further fractionated on a sepharose gel column.

Column: 300 x 9 mm, packed with sepharose CL-4B (preserved with 20 % ethanol), buffered and eluted with 0.01 M sodium dihydrogen phosphate solution at pH 8. Amphotericin B (250 µg / ml) was added to the buffer solution to protect against microbial growth.

Flow rate: 8 ml per hour.

Detection wavelength: Proteins were detected photometrically with Bradford reagent at 580 nm, carbohydrates were detected with PAS reagent at 550 nm.

Bradford reagent to detect proteins

stock solution:	100 mg Coomassie Blue G, 50 ml ethanol (97 % v/v), 100 ml H_3PO_4 (85 %); temperature during storage: 4 °C;
solution for usage:	3 ml stock solution, 17 ml aqua dest.;
detection:	50 µl sample are detected by 200 µl reagent on a covered microtitre plate after 10 min. at room temperature
detection wavelength:	580 nm.

PAS method to detect carbohydrates

chemicals:	fuchsin, periodic acid, acetic acid (7 %), sodium pyrosulphite, 1 M HCl, activated charcoal;
periodic acetic acid:	10 mg periodic acid are added to 10 ml acetic acid (7 %)
Schiff reagent:	1 g alkaline fuchsin is solved in 100 ml aqua dest., brought to the boil, left to cool at ca. 50 °C, and 20 ml 1 M HCl and ca. 500 mg activated charcoal are added. This suspension is stirred for about 10 min. and filtrated twice. A clear deep red coloured solution is obtained. To decolourise the Schiff reagent 100 mg sodium pyrosulphite are added to 6 ml Schiff reagent and left to stand for about 2 hours in a water bath at 37 °C.
detection:	50 µl periodic acetic acid was added to150 µl sample on a microtitre plate, which is incubated at 37 °C for about 2 hours (the time until the Schiff reagent is decolourised). Then 50 µl decolourised Schiff reagent are added to each sample. The covered plate is left to stand for 30 min. at room temperature.
detection wavelength:	550 nm

Compositional analyses of carbohydrates and amino acids

Aliquots of the pooled mucilage fraction were hydrolysed, lyophilised and trimethylsilylated prior to amino acid and carbohydrate compositional analyses. These GC analyses were performed at the laboratory of Dr. Kownatzki and Dr. Blaskowitz, MHH.

To determine the amino acid composition, the plant mucilage was hydrolysed with 6 N HCl for 24 hours at 110 °C. After drying in a Speed Vac centrifuge, samples were trimethylsilylated with MTBSTFA.

To determine the monosaccharide composition, hydrolysis was performed with 0.5 M methanolic HCl for 15 hours at 75 °C. Volumes were reduced by Speed Vac centrifuge and trimethylsilylation was performed with BSTFA.

Determination of the dry residue:

Samples of respectively 200 µl were reduced in volume by means of a Speed Vac centrifuge and dried overnight on P_2O_5 and NaOH *in vacuo* at 70 °C until constancy of weight.

GC-conditions:
AEI MS30 74; column: SE 54; helium; 2 °C per minute; temperature to determine the saccharides: 70-300 °C; temperature to determine the amino acids: 100-320 °C.

Data collection: Kratos DS 55.

Linkage analysis (= methylation analysis) and partial hydrolyses

Linkage analysis
To determine linkages between aldoses (here aldohexoses), aliquot solutions containing the intact polysaccharide were permethylated, hydrolysed, and reduced by means of $NaBH_4$ and peracetylated. To determine uronic acids (here hexuronic acids), samples were permethylated, reduced by means of $NaBD_4$, hydrolysed, reduced by means of $NaBH_4$, and peracetylated.

By the permethylation procedure all free hydroxyl groups of the intact polysaccharide are methylated yielding methyl ethers. The subsequent hydrolysis yields monosaccharide units with free hydroxyl groups at positions that had formerly been linked to further sugar units, while the methyl ether groups remain unaffected. Reduction with $NaBH_4$ leads to conversion of aldoses (here aldohexoses) to their corresponding alditols (here only aldohexitols). So, in hexopyranoses, the aldehyde group at the enantiomeric carbon is reduced to an alcohol and therefore ring opening occurs between C1 and C5 yielding a linear structure. Subsequent peracetylation marks the position of ring fission (C1 and C5 of hexopyranoses) and in addition all positions where sugar components had been attached, which is the major point of interest within the methylation analysis. Thus, open chained sugar alcohols are generated from former aldoses bearing methoxy or acetoxy groups at each carbon atom (see App. II.5).

In case of hexuronic acids, permethylation yields methoxy groups as described for the aldohexoses and one additional methyl ester function at position C6, the former carbonic acid. As in contrast to free carbonic acids, ester groups can be reduced with metal hydrides, reduction is now performed by means of $NaBD_4$, leading to conversion of hexuronic acids to the corresponding hexose derivative with two deuterium atoms incorporated at C6 position ($-CD_2OH$: isotope tagging). Thus, it becomes possible to distinguish between position 6 of a former hexuronic acid and the respective hexose. After subsequent hydrolysis to get free monosaccharides, a further reduction step follows, with $NaBH_4$ to convert the aldehyde function at position C1 (anomeric carbon atom) into an alcohol function as described for aldohexoses. Finally, peracetylation yields acetoxy groups at C1, C5, C6 and all hydroxy groups to which another sugar moiety had been formerly attached.

The methylation analysis was carried out at the laboratory of Dr. M. Nimtz (GBF, Braunschweig). The procedure was performed according to Nimtz *et al.* (1996), which is based on the method of Hakomori (1964).

Methylation of the unsubstituted hydroxyl groups of all sugar components was achieved using the methylsulfinyl carbanion as catalysing base, the conjugate base of dimethyl sulfoxide (DMSO), generated by dissolving a weighed amount of sodium hydride in DMSO. Excess of methyl iodide was added as methylation reagent. Both, the amount of the methylsulfinyl carbanion and the sodium hydride have to be nearly equivalent to the content of hydroxyl groups expected. The reaction was carried out as one continuous process (Hakomori, 1964).

A weighed sample of plant mucilage was dissolved in 0.2 ml of DMSO (magnetic stirrer) at slightly elevated temperatures (40-50 °C) under a stream of nitrogen. Under continuous stirring, equal amounts of the methylsulfinyl reagent were added, and finally a 10-fold excess of methyl iodide was added. The reaction mixture was diluted with water and the methylated products were extracted by means of lipophilic solvents, which were in turn purified by gel chromatography on Sephadex LH20 to remove contaminants of low molecular weight.

Reduction with $NaBD_4$ to discern hexuronic acids from native hexoses:

200 µl of a solution of 5 mg $NaBD_4$ in 1 ml 1 % (v/v) ammonia solution in water was added to the permethylated polysaccharide. To increase solubility, 200 µl of ethanol were added. The reaction mixture was then left to stand overnight. The reaction was interrupted by one drop of acetic acid; after evaporation of the solvents, the boric acid generated *in situ* was removed by means of 5x evaporation of the sample dissolved in 200 µl of 1 % (v/v) methanolic acetic acid.

The reduction with $NaBD_4$ is omitted for native aldohexoses.

Hydrolysis was performed with 4 N TFA for 2 hours at 100 °C.

Reduction with $NaBH_4$ to reduce the aldehyde function of aldohexoses (including aldohexuronic acids) at the anomeric carbon atom to the respective alcohol (alditol):
1 mg $NaBH_4$ was added per ml sample solution, other conditions were as described for step two.

Peracetylation: 100 µl of acetanhydride plus one drop of acetic acid were added to each sample, which was kept for 2 hours at 100 °C.

For detection GC-MS and ESI-MS/MS analyses were used.

Partial hydrolyses
Polysaccharide samples of the leaf mucilage were partially hydrolysed with trifluoroacetic acid at three different concentrations. Each of the resulting oligosaccharide mixtures was reduced by means of $NaBD_4$ to mark the reduced position C1 from position C6 of former aldoses by introduction of one deuterium atom at the anomeric C1 (-CHDOH). It should be noted that this time reduction with $NaBD_4$ only leads to a reduction of the aldehyde function at the anomeric carbon atom of hydrolysed hexose and hexuronic acid moieties, while the carbonic acid function at C6 of hexuronic acids remains unaffected.

This step allows for a differentiation between position 3 and position 4 in case of substituted hexoses, as otherwise fragments of like masses would have been produced during mass spectrometric fragmentation of the derivatives. Subsequent permethylation yielded methyl ethers of all hydroxyl functions and a methyl ester at the former carbonic acid group, respectively. Finally, these derivatised oligosaccharide-alditol-1-*d* samples were directly analysed by means of EI-MS (GC-MS and subsequent MS/MS) and positive ESI-MS (and subsequent MS/MS).

Thus, samples containing native polysaccharides were partially hydrolysed with 0.1 N, 1 N and 4 N TFA for 1 hour at 100 °C, respectively. Subsequently, samples were then reduced with $NaBD_4$ and permethylated according to procedures as described for the methylation analysis. After purification on Sephadex LH20, samples were directly analysed by GC-MS/MS and ESI-MS/MS.

Detection by mass spectrometry
Experiments were performed according to Nimtz *et al.* (1997).

GC-(EI)MS and MS/MS experiments were performed on a Finnigan GCQ ion-trap mass spectrometer (Finnigan MAT Corp.) running in the EI mode equipped with a 30-m DB5 capillary column. It was started at a temperature of 80 °C, 2 minutes were run isothermally, then it was heated 10 °C / min. until 340 °C were reached. MS/MS experiments on this instrument were performed by isolating the respective parent ions in the trap and setting the collision energy to about 0.7 eV.

ESI-MS/MS analyses were performed on a Finnigan MAT TSQ 700 triple-quadrupole mass spectrometer equipped with a Finnigan electrospray ion source (Finnigan MAT Corp.). The reduced and permethylated samples were dissolved in acetonitrile, saturated with NaCl (approximately 10 pmol / µl) and injected at a flow rate of 1-3 µl / min into the electrospray chamber. In the positive-ion mode, a voltage of +5.5 kV was applied to the electrospray needle. For fragmentation of the sodium adducts, 40-70 eV were applied to the collision cell.

3 Results

3.1 Swelling number and gelling properties

To characterise the gelling properties of leaf species eaten by the Coquerel's sifakas, the "swelling number" was determined for all plant species collected during the study times at Duke (Tab. C.19). Although normally triple assays have to be performed, in particular for *A. julibrissin* leaves and Plantaginis ovatae testa more tests were carried out.

Tab. C.19: Averaged swelling number of plant specimens.

plant sample	May	July	October
Acer rubrum	7		6
Albizia julibrissin	9	12-18	9[a]; 10[b]
Cercis canadensis	8		12
Liquidambar styraciflua	7		8
Liriodendron tulipifera			10
Photinia glabra[c]			8
Plantaginis ovatae testa			54
Rhus copallina	7	8	7
Robinia pseudoacacia	7		8
Vitis rotundifolia			8

[a] younger leaves

[b] older leaves

[c] Due to insufficient amounts of plant material of *Photinia glabra* only simple tests could be performed (n=1).

Except for the Plantaginis ovatae testa as reference compound, the highest swelling number was found for *A. julibrissin* leaves harvested in July. Although other plant specimens also showed in part high numbers, only powdered *A. julibrissin* leaves produced a green gel if the leaf material was soaked in water.

The Plantaginis ovatae testa material was of premium quality and extremely fine. Hence, the required soaking number of 40 (DAC 1986) was clearly exceeded. In comparison, according to DAB 9 intact Psyllii semen had a swelling number of 10, and for Plantago ovatae semen a swelling number of 9 was prescribed.

Dosage calculations for the amount of plant mucilage eaten by the Coquerel's sifakas (see chapter C.VI) was based on the swelling number. The swelling number of 9 was used for the leaves ingested in May and the younger autumn leaves, respectively, since for these months feeding data were recorded. It should be remarked that storage conditions may have impact onto the swelling number so that the swelling number for May leaves may be determined somewhat low, and a possible difference to the swelling number of younger autumn leaves may not become visible.

Gelling properties in the presence of tannins

Principally during the spring months, *R. copallina* and *A. julibrissin* leaves were ingested within one meal time in substantial amounts by the focal sifakas. Hence, the question arose if the chemical leaf compounds, respectively the mucilage of *A. julibrissin* and the gallotannins of *R. copallina* leaves, would interact negatively or positively. Viscous solutions and gels are often disturbed by tannins due to incompatibilities based on differing pH optima. As a result, various gel preparations from *A. julibrissin* leaves with *R. copallina* leaves or *R. copallina* leaf extracts were used to examine this problem.

a) First, a simple gel was prepared with *A. julibrissin* leaves, whereby some powdered *R. copallina* leaves were added. The gel became significantly stiffer and did not break. With the addition of tannins extracted from *R. copallina* leaves (C.IV.2.5) to the simple *A. julibrissin* gel, similar results were obtained.
b) Another experiment was performed using emulsions prepared with paraffin (Mital and Adotey, 1973) which could easily be stirred into the gel. Adding the dispersion of *R. copallina* leaf tannins in paraffin to the *A. julibrissin* gel emulsion, resulted once again in a smooth and stiff gel.
c) Measurements of the pH corroborate the finding that *A. julibrissin* gel is obviously compatible with tannins. While a 4 % (m/v) gel of *A. julibrissin* leaves yielded a pH of about 4, Ispaghula husk gel showed neutral pH (about 7). Addition of some commercial tannic acid to Ispaghula husk gel lowered the pH to 1-2. In contrast, the addition of tannic acid to 4 % and 8 % (m/v) *A. julibrissin* gel preparations yielded a pH of approximately 3. Obviously, *A. julibrissin* gel possesses a buffering capacity to some extent and its acidic properties allow for a compatibility with tannins, which is not the case for neutral mucilages such as preparations with Plantaginis ovatae testa.

3.2 Chemical composition of *A. julibrissin* leaf mucilage

Polysaccharide fractions of high molecular weight were collected from *A. julibrissin* leaf samples by gel filtration. These fractions contained polysaccharides with a molecular weight of approximately 2 million Dalton and were used to chemically characterise the leaf mucilage. Guided by detection of carbohydrates with PAS reagent, 40 fractions of 1.3 ml each were collected, from which fractions 10 to 18 containing high molecular weight polysaccharides were pooled.

To investigate the composition of monosaccharides and amino acids of *A. julibrissin* leaf mucilage, aliquots of the high molecular weight fractions were hydrolysed and the resulting monosaccharide and amino acid components were determined by GC analyses. The dry residue yielded 4.2 % carbohydrates and 0.4 % amino acids.

The main monosaccharide components were rhamnose (38 %), galacturonic acid (29 %) and galactose (33 %), that is a molar ratio of nearly 1:1:1. Monosaccharides found during the subsequent linkage analysis (= methylation analysis) corroborated the occurrence of rhamnose, galactose and galacturonic acid as major monosaccharides. Amino-groups containing sugars were not found.

The following amino acids were detected within the fraction of proteins: glutamic acid (17 %), aspartic acid (11 %), alanine (10 %), serine (10 %), and leucine (10 %) were found by at least or more than 10 %. Valine (8 %), glycine (6 %), isoleucine (5 %), lysine (5 %), tyrosine (5 %), threonine (4 %) and phenylalanine (4 %) occurred by less than 10 %, respectively. Proline (2 %) and methionine (1 %) appeared as minor components. Although the type of binding between the portion of polysaccharides and the portion of proteins was not further investigated in this work, at least in part covalent bonds are assumed to appear between both like in most gums and mucilages (Hegnauer, XIa, 1994).

According to the identification and quantification of the major components of *A. julibrissin* leaf mucilage in this study, *A. julibrissin* leaf mucilage can be classified as acidic and pectin-like mucilage.

In contrast, the mucilaginous polysaccharides from *Plantago* seeds, such as the drug Plantaginis ovatae testa (used as reference compound in this study), belong predominantly to the water-soluble fibre type (hemicelluloses), and consist basically of arabinoxylans (Rimpler, 1999). According to the scientific information of Dr. Falk Pharma GmBH on Plantaginis ovatae testa, the husks consist of 85 % mucilaginous polysaccharides, which in turn contain xylose (63.6 %), arabinose (20.4 %), rhamnose (6.4 %) and galacturonic acid (9.4 %) as major components.

Linkage of the sugar moieties of the mucilage by methylation analysis
A set of complementary techniques was used for a preliminary structural characterisation of the polysaccharide isolated from *A. julibrissin* leaf mucilage. Whereas carbohydrate compositional analysis after total hydrolysis of the polysaccharide revealed the identity of its monosaccharide constituents, the linkage type of individual monosaccharide units was elucidated by the methylation analysis. The sequence of monosaccharide units in the carbohydrate chain and the detection of branching points was achieved by GC/MS analysis of small reduced and permethylated oligosaccharides and the MS/MS analysis of larger oligosaccharide units obtained after partial hydrolysis of the oligosaccharide chain. Complementary aspects of these data allow for a rough description of the overall structural patterns to be found in the native polysaccharide. But not all structural parameters like anomericity of the monomers, the exact definition of branching points, the "decoration" of the native oligosaccharide chain with acetyl groups etc. could be unambiguously solved by the methods applied.

To determine the linkages between the major monosaccharide units, a methylation analysis was performed with subsequent GC-(EI)MS analysis.

In this study, two types of linkages have been demonstrated for rhamnose moieties, that is 1,2- and 1,2,4-linked rhamnose (deoxymannose) moieties with the largest peak in the GC-spectrum originating from the 1,2-linked rhamnose. For galactose and galacturonic acid 1,4-linkages were found, respectively. For details see App. II.5.

The sequential analyses of oligosaccharide units were performed after partial hydrolyses, reduction and permethylation followed by GC-MS/MS and ESI-MS/MS detection.

Results showed that 4 N TFA was superior to lower concentrated TFA for the partial hydrolysis of the polysaccharide contained in the mucilage.

With respect to mass spectrometry the positive ESI-mode was preferred and gave valuable information generating sodium adducts of the hydrolysed oligosaccharide units (App. II.5).

The sequential analysis yielded disaccharide units consisting of galacturonic acid, which was bound to deuterium labelled rhamnitol, and galacturonic acid being attached to deuterium labelled galactitol, respectively. Based on results of the methylation analysis and fragments generated through EI-MS experiments, it is concluded that these disaccharide units are composed of GalA 1→ 4 Rha-ol-1-*d* and GalA 1→ 4 Gal-ol-1-*d* units, respectively (see App. II.5). As by linkage analyses only 1,2- and 1,2,4-linked rhamnose has been found, it can be assumed that during partial hydrolysis obviously the 4-linkage of the rhamnose was more resistant to hydrolysis conditions than the 2-linkage, which was obviously cleaved before methylation. So, it can be assumed that the GalA 1→ 4 Rha unit originates from a branched 1,2,4-linked rhamnose moiety which has originally been further substituted in 2-position.

Furthermore, after partial hydrolyses the presence of several linear sequences and one branched sequence could be shown by applying the EI-MS and ESI-MS techniques.

The linear oligosaccharides were found to consist of four (tetramer), six (hexamer) and even eight (octamer) saccharide units with alternating galacturonic acid and rhamnose moieties, the latter exhibiting a molecular weight of 1631 Dalton. For the sequence of the linear oligosaccharides the following structure can be proposed, with $[]_n$ as repeating disaccharide unit: GalA 1→ 2 Rha 1→ [4 GalA 1→ 2 Rha 1]$_n$ → 4 GalA 1→ 2 Rha.

From the total molecular weight of the polysaccharide of 2 Mio Da, it can be calculated that, on average, n must be larger than 1000. Although the 2-linkage of the rhamnose moieties within the chain could not be unambigously proved by EI experiments, it is reasonable to assume 1,2-linked rhamnose moieties, as after methylation analyses the largest peak in the GC-spectrum originated from1,2-linked rhamnose.

A branched structure could be found for a tetrasaccharide unit with galactose as third major component (Fig. C.12). Although the type of linkage between the galacturonic acid and the initial rhamnose residue was found to be GalA 1→ 4 Rha by fragments generated through EI-MS experiments, attachments of the sugar moieties to the C-2 and C-4 of the initial branched rhamnose residue cannot be unambigously assigned.

^{1}HexA
|
O
|
HexA^{1}-O-^{4}Hex^{1}-O-^{2}dHex^{4}-ol-1-*d*

Fig. C.12: Proposed structure for a branched tetrasaccharide unit. Types of linkage are based on methylation analyses, and may be reversed at the branched site.

Galacturonic acid always built the terminal end, while rhamnose built the initial reduced moiety. As galacturonic acid has never been found to occur in the reduced form, it can be assumed that the linkage between galacturonic acid and rhamnose was more stable than the linkage between rhamnose and galacturonic acid.

Hence, *A. julibrissin* leaf mucilage, which has been investigated for leaves from May, can be classified as pectic-like polysaccharide, a rhamnogalacturonan with a hypothetical backbone structure in which galacturonic acid and rhamnose moieties occur as alternating units. In branched regions that also contain galactose moieties as third major component, 1,2,4-linked rhamnose appeared as the site of ramification. Galacturonic acid was found as terminal side chain component.

4 Discussion

The mucilaginous polysaccharides in *A. julibrissin* leaves found in this study were characterised by compositional and linkage analyses to determine its chemical structure (App. II.5) and by differing physico-chemical experiments to assess its possible suitability for medicinal purposes.

The structure of alternating 1,4-linked galacturonic acid and 1,2-linked rhamnose residues were the backbone structure of rhamnogalacturonans with 1,2,4-linked rhamnose residues as the site of ramification, which agrees with hairy regions in pectins as part of the cell wall structure and pectin-like polysaccharides as fractions from plant mucilages, which occur in different species throughout plant kingdom (Stephen, 1983 (review); Kiyohara *et al.*, 1988; Müller *et al.*, 1989, Yamada *et al.*, 1990; 1991; An *et al.*, 1994; Schols *et al.*, 1994; 1995; Vignon and Garcia-Jaldon, 1996; Rimpler, 1999).

Schols *et al.* (1994; 1995) isolated rhamnogalacturonan oligomers from hairy regions of apple pectin consisting of 4 to 9 sugar units with a backbone of alternating galacturonic acid and rhamnose residues. Despite different analytical methods (compared to this study), some of the oligomers found by Schols *et al*. (1994; 1995) perfectly agree with structures proposed for the high molecular weight fraction of *A. julibrissin* leaf mucilage. Structures similar to the hairy regions of apple pectin were also found in various fruits and vegetables like potato fibre, carrot, onion, leek, grape and pear tissue (Schols *et al.*, 1994; 1995).

Pectin (Pectinum, ÖAB) itself is a heteroglycan, containing unbranched homogalacturonane regions, so called smooth regions, which are interrupted by rhamnogalacturonan regions carrying neutral carbohydrate side chains. These branched regions are called “hairy regions”. While homogalacturonan regions contain α-1,4-linked galacturonic acid residues, rhamnogalacturonan regions consist of α-1,4-linked galacturonic acid and α-1,2-linked rhamnopyranosyl residues substituted with neutral side chains as arabinans, galactans, or arabinogalactans attached to O-4 of the rhamnopyranosyl residue. In homogalacturonan regions carboxyl groups are in part methylated and hydroxyl groups acetylated, respectively, while in rhamnogalacturonan regions only hydroxyl groups carry in part, acetic acid ester. In some pectins like apple pectin, homogalacturonan regions may be substituted by xylogalacturonans with xylose residues being attached to the O-3 of galacturonic acid residues (Rimpler, 1999).

Hence, the high molecular weight fraction of *A. julibrissin* leaf mucilage can be characterised as rhamnogalacturonan in agreement with hairy regions in pectins and pectin-like polysaccharides. However, in contrast to most pectins and pectic polysaccharides, in this study no pentoses such as arabinose or xylose could be found as neutral side chain components.

Of the large number of plant gums that have been examined chemically, the majority are substituted arabinogalactans, with most containing uronic acids and rhamnose as well (Stephen, 1983). Focusing on the Mimosaceae, especially gums of the genera *Acacia* and *Albizia* have been most extensively studied with gum Arabic from *Acacia senegal* and further *Acacia* species as prominent example.

Thus, gums from more than 60 species of *Acacia* (Anderson and Stoddart, 1966; Anderson and Karamalla, 1966; Anderson and Cree, 1968a and b; Anderson *et al.*, 1968; Anderson and Dea, 1969; Anderson *et al.*, 1983; Stephen, 1983; Anderson *et al.*, 1984; Martínez *et al.*, 1992; Rimpler, 1999) and 11 species of *Albizia* (Anderson *et al.*, 1966; Mital and Adotey, 1971; Stephen, 1983; Anderson and Morrison, 1990; Martínez *et al.*, 1995) have been most extensively investigated. The number of studies on *Acacia* gums are impressive, the number of known species exceeds 900 (Stephen, 1983), while the genus *Albizia*, tribe Ingeae, comprises 150 species (Anderson and Morrison, 1990). However, species-specific differences are significant (Stephen, 1983; Delgobo *et al.*, 1998).

As to chemical peculiarities, gums from investigated *Albizia* species are reported to contain mannose, which does not occur in *Acacia* gum exudates (Anderson and Morrison, 1990; Hegnauer, XIa, 1994). Depending on the species, *Albizia* gums consist of 1-16 % 4-O-methylglucuronic acid, which could not be found in this study, 5-29 % glucuronic acid, 14-55 % galactose, 1-13 % mannose, 14-40 % arabinose and 7-29 % rhamnose (Anderson *et al.*, 1966; Anderson and Morrison, 1990). For *Albizia zygia* gum, galactose, arabinose, glucuronic acid and galacturonic acid were found as components (Mital and Adotey, 1971). In a later study on *Albizia lebbeck* (Martínez *et al.*, 1995), which is also mentioned as gum Arabic substitute, no rhamnose residues have been found. However, Hegnauer (2001) does state that the monosaccharide components of Mimosaceae hetreopolysaccharides largely agree, as most contain 4-O-methylglucuronic acid, glucuronic acid, galactose, arabinose and rhamnose.

However, in contrast to the aforementioned studies, which cite both *Albizia* and *Acacia* gums as gum exudates from trunks, stems or branches, the polysaccharide of this study is a leaf mucilage and quite different in its chemical composition from all other *Acacia* and *Albizia* gums described so far. However, in this study's analysis of *A. julibrissin*, mannose occurred in trace amounts, which was evidenced in the GC spectrum during the compositional analysis. This corroborates the occurrence of this monosaccharide in the genus *Albizia*.

Previously, the only hint to the occurrence of polysaccharides in *A. julibrissin* is given by Moon *et al.* (1985), who investigated crude polysaccharides containing extracts of various medicinal plants. Although a certain antitumor activity was found for the crude fraction of polysaccharides from *A. julibrissin*, neither the exact plant tissue used, nor any characteristics on the chemical composition of the polysaccharide were reported. Accordingly, the acidic pectin-like mucilage in the leaves of *A. julibrissin* is first characterised in this study.

As to the amino acid composition of *Albizia* gum exudates, aspartic acid and hydroxyprolin appear to be the major components in most species, with proline often as third important amino acid (Anderson and Morrison, 1990). Subsequent discussion will note that proline and hydroxyprolin are important amino acids, and are able to bind to tannins. However, in this study, the content of proline in *A. julibrissin* leaf mucilage is relatively low, while the presence of hydroxyprolin could not be detected.

As to physico-chemical properties, a swelling number of 9 has been determined for *A. julibrissin* leaves of May and younger October leaves (Tab. C.19). Although the July leaves exhibited much higher swelling numbers, these values have not been used for dosage calculations (see chapter C.VI), as no feeding data had been recorded during the month of July. Although no direct relation appears to exist between the swelling number of a drug and its suitability in medicine, (e.g. its use as filling, water-binding and antidiarrhoeal agent), the swelling number is used in this work as a marker to calculate therapeutic dosages and to compare *A. julibrissin* leaf mucilage's numbers to the swelling numbers and dosages of other antidiarrhoeal drugs.

Despite high swelling values for other plants growing in the forest (see Tab. C.19), the gelling polysaccharides of high molecular weight were only identified in *A. julibrissin* leaves. In this context it should be mentioned that dried leaves may contain mucilage of a different, albeit a lower quality than fresh leaves (Bräutigam and Franz, 1985a and b).

The acidic pH of aqueous dispersions of ground *A. julibrissin* leaves is thought to be responsible for its compatibility with tannins. While normally occurring tannins disturb gelling agents as evidenced in the Ispaghula husk gel, this was not the case for the *A. julibrissin* leaf mucilage. *A. julibrissin* gel became stiffer in the presence of hydrolysable tannins while Ispaghula husk gel became more liquid.

The observed buffering capacity of *A. julibrissin* gel might also have an impact on physiological processes within the gut, e.g. bacterial growth. Furthermore, as evidenced for pectin (Janssen and Queisser, 1982), a dependence of the gel viscosity on pH and temperature (highest viscosity of pectin at pH 7.2 and gel thinning with increasing temperature) which may also be expected for *A. julibrissin* leaf mucilage, with an important influence on the gastrointestinal microflora and motility. According to Anderson and Morrison (1990) some *Albizia* species have a much higher intrinsic viscosity and higher rhamnose contents than any recorded *Acacia* gum.

In comparison, for a 2 % (m/v) mucilage of *Albizia zygia* gum a pH of 4.8, for *Acacia* mucilage a pH of 4.3 was reported (Mital and Adotey, 1971). *Albizia zygia* gum exhibited the highest viscosity at pH of approximately 5. Lower and higher pH values decreased viscosity significantly. Furthermore, gel viscosity decreased with temperature and aging (Mital and Adotey, 1972 and 1973). The gum exhibited pseudoplastic flow with thixotropy (Mital and Adotey, 1972).

Plantago seed mucilages are reported to yield thixotrope gels at equal or higher concentrations than 2 % (Rimpler, 1999).

Finally, the view should be extended to food plants occurring in Madagascar and the question whether plant polysaccharides are known as secondary plant compounds to *Propithecus* species. There are numerous indications that within their natural Malagasy habitat, *Propithecus* species utilise gum or mucilage producing trees as food plants (Ranarivelo, pers. comm.; Richard, 1978a; Meyers, 1993), although gum feeding has not been reported for *Propithecus* species until now. However, species of the Combretaceae, especially the genera *Combretum* and *Terminalia* (Anderson and Bell, 1976; Anderson and Bell, 1977; Stephen, 1983; Hegnauer, 2001), species of the Burseraceae (Stephen, 1983; Hegnauer 2001) and in particular, species of the Mimosaceae (genera *Albizia* and *Acacia*, see above) are known to produce gum exudates or incorporate plant mucilages, yet some of these aforementioned plants are reported to be food plants by wild populations of *Propithecus*. Thus, the role of plant polysaccharides as secondary plant compounds in food items selected by *Propithecus* species in the wild needs more appreciation. In this light the use of *Albizia lebbeck* by *P. tattersalli* raises the question of possible “gum feeding”.

C.VI Pharmacological effects of the food plants analysed

1 Introduction

The major health problem of captive *P. v. coquereli* has been a history of acute and chronic diarrhoea prior to conditions whereby they had access to leaf species of their liking. (Haring, 1988; Spelman *et al.*, 1989). Phytochemical investigations in this study have been conducted on the most preferred food plants, *Rhus copallina* and *Albizia julibrissin*, and the resulting analysis revealed the occurrence of three major groups of secondary plant compounds: hydrolysable tannins, flavonoids with predominantly quercetin as aglycone, and a pectin-like plant mucilage (chapters C.III - C.V).

For ethical reasons, it was not approved by the Duke Lemur Center Research Council to conduct feeding choice experiments with the sifakas. But the question was raised, as to why the focal lemurs selected these special plant species and their specific plant parts. The analytical data of the food plants and their inherent secondary compounds (see chapters C.III - V) combined with pharmacological *in vitro* assays described in this chapter, together with the symptoms described in the necropsy reports of dead sifakas will be fully utilised to assess this central question.

In this chapter the three analysed major groups of secondary plant compounds will be investigated for possible antimicrobial and enterotoxin inhibiting activities in the context of diarrhoea.

Thus, various leaf extracts and preparations of *R. copallina* and *A. julibrissin* leaves, and in part, various other food plants, were tested against different bacterial strains, one protozoal and several fungal strains. As hydrolysable tannins are subject to enzymatic hydrolysis in the digestive tract, they yield gallic acid monomers, and gallic acid itself has been included in some antimicrobial assays within this study. Based on these results, *R. copallina* leaf extracts were tested additionally for possible enterotoxin inhibiting properties. For this assay, cholera toxin was chosen, which is known to cause massive diarrhoea in humans.

In view of the large amounts of plant material ingested by the sifakas, the question of relevant therapeutic dosages of their inherent major plant compounds, and dosage-effect relationships will be discussed in comparison to known antidiarrhoeal phytotherapeutics and their recommended dosages within human medicine. Pharmacological interactions between these groups of secondary plant compounds and their possible effects on the gastrointestinal integrity will be discussed. The necropsy reports describing symptoms that were associated with the death of captive sifakas will be considered in the light of pharmacological effects inherent to these groups of chemical plant compounds. It appears that these compounds were not sufficiently supplied by the animal’s food until the dietary regime was revised.

Diarrhoea in captive sifakas is an ongoing problem, especially when the food supply fails the needs of the sifakas. Diarrhoea in human medicine is defined by more than three daily emptyings of the gut, a diminished stool consistency with more than 90 % water content and a stool weight of over 250 g per day. Accompanying symptoms may be abdominal pain, nausea, emesis, fever and / or haemorrhages. The acute diarrhoea is self limiting after 3-4 days, while a chronic low grade diarrhoea may persist for more than two weeks (Viegener, 1994; Dingermann and Loew, 2003).

Diarrhoea is a symptom based on a variety of causes and is not a disease in itself. If no specific cause can be found, diarrhoea can be classified as unspecific. While a chronic diarrhoea is essentially non-infectious, acute diarrhoea may be infections linked with viruses, bacteria, bacterial toxins and / or parasites. In developing countries, toxin producing bacteria such as the cholera toxin are one of the main causes of diarrhoea with the highest mortality rates in children under five years of age (WHO, 1999 and 2000).

Already existing diseases such as chronic inflammatory bowel diseases (e.g. Crohn's disease, ulcerative colitis (Elson *et al.*, 1995; Kruis *et al.*, 2003)), tumours, organic causes such as hyperthyreosis, the syndrome of malabsorption, together with malnutrition (Sepúlveda *et al.*, 1988; Bhandari *et al.*, 1996; Prasad, 1998), food intolerance, allergies, physical and psychological phenomena, or undesired side-effects of pharmaceuticals (like antibiotics) are possible reasons for non-infectious diarrhoeas. Viruses (e.g. Rotaviruses), bacteria and / or their toxins, such as enterotoxin producing *E. coli* (traveller's diarrhoea; Raedsch *et al.*, 1991), *Vibrio cholerae*, *Staphylococcus aureus*, *Shigella* and *Salmonella* species, *Clostridium difficile*, *Cl. perfringens* and *Cl. botulinum*, *Campylobacter jejuni*, *Yersinia enterocolitica* (Bresnahan *et al.*, 1984), and parasites such as Protozoa (amoeba, *Giardia lamblia*) and parasitic worms are reasons causing acute infectious diarrhoeas (Thorne, 1986; Dingermann and Loew, 2003), albeit their underlying mechanisms and severity for the host animal can vary considerably (Thorne, 1986). Based on different pathophysiological mechanisms, another division of diarrhoeas is known as diarrhoea of malabsorption (osmotic diarrhoea), a secretory diarrhoea and a functional diarrhoea (Dingermann and Loew, 2003).

Generally, the therapy to correct diarrhoea depends on its causation, but overall the cure is serving to ameliorate its symptoms. For an acute and especially unspecific diarrhoea rehydration and (antidiarrhoeal) therapeutics are called for (Ruppin, 1994), albeit more specific antiviral or antibacterial measures may be necessary. Antidiarrhoeal phytotherapeutics are often used for acute and chronic diarrhoeas because of their therapeutic broadness, low or nearly absent toxicity and their "unspecific" mode of action exhibiting many different desired effects (Dingermann and Loew, 2003; Rote Liste, 2005)

While studying the **necropsy reports** of the Coquerel's sifakas from the Duke Lemur Center and from the San Diego Zoo (Spelman *et al.*, 1989) (most animals had been wild-caught from Madagascar), two general underlying courses of pathogenesis could be determined. The sifakas had developed an acute and devastating diarrhoea, followed by death, or they may have died from multiple infections including septicaemia following a history of chronic and recurrent diarrhoea. Both, specific and unspecific diarrhoeas had occurred and in part, the necropsy reports resembled each other in symptoms. [In the following cases, only the most salient features of the maladies will be discussed.] In cases of accelerated deaths, associated findings were e.g. necrotic enteritis, renal failure, salmonella infection, pneumonia and gastritis (Spelman *et al.*, 1989).

In cases with a history of chronic recurrent diarrhoea (Duke Lemur Center), animals eventually developed in some cases, a protozoal superinfection, a haemorrhagic pancreatitis, a wasting syndrome, or a septicaemia with a lethal sickness. The protozoal superinfection has been described as multifocal necrosis with protozoal organisms while the heart, liver and spleen, had acute interstitial pneumonia with protozoal organisms and oedema, multifocal myofiber atrophy with interstitial fibrosis in the heart, focal, ulcerative enteritis. A diagnosis of septicaemia was described with findings of a vasculitis, vascular thrombosis, necrotising interstitial pneumonia, myocardial necrosis and enlarged heart, hypertrophy of the pyloric muscle and mild and diffuse lipidosis. At least one septicaemia was caused by *Yersinia enterocolitica*. One sifaka died from diabetes, another from adenocarcinoma in the small intestine. At the San Diego Zoo, one Coquerel's sifaka (the others had died within one week of importation to the zoo) developed a hemosiderosis. Furthermore, it is notable that a high frequency of dental diseases occurred, such as broken incisors, submandibular swelling or oedema (Duke Lemur Center).

An alteration in primary food composition already improved health conditions of captive sifakas at the Duke Lemur Center before the onset of this study (milk products such as cheese, eggs, cereals and bread, as well as products too rich in simple saccharides such as honey and juice, had been eliminated from the diet). Furthermore, it is well acknowledged that dietary changes affect the gastrointestinal microflora and the absorbing processes of essential nutrients, especially in respect to diarrhoeal incidences of those with water and electrolytes (Stevens, 1988). Considering the human condition, an overload of simple saccharides over a protracted period of time favours the generation of caries, diabetes, adiposis and related metabolic diseases. Depending on the type of mono- and disaccharides and absorptive processes they rely on, an overload of these compounds may cause osmotic diarrhoea (Gray, 1984). Dietary imbalances of this type in this respect may have favoured the genesis of e.g. diabetes of previously captive sifakas.

However the introduction of the browse contributed significantly to improved health of the animal followed by successful breeding with the noticeable lack of the "traditional winter slump" (Haring, 1988). It should also be noted that before having access to desired leaf species, the sifakas often died despite anti-infective and rehydrating therapies. This indicates, that the secondary leaf compounds may play a preventive character, to keep the sifakas healthy. In another line of discussion, curative effects may be attributed to the major leaf compounds.

To explain the courses of diseases leading to these lethal episodes for the sifakas, some additional explanations on the **intestinal milieu** and their physiological and pathological processes need to be elucidated.

Normally, the gastrointestinal microflora is within an ecological equilibrium and relative constant of a healthy person, yet there are significant influences from physical, biological, chemical, environmental and host factors. Physiologically, the microflora changes with age, and pathologically, such as in diseased persons, e.g. with chronic inflammatory bowel diseases (Onderdonk, 1998; Ahn *et al.*, 1998). Therefore, the state of the microflora depends on the genesis and formation of various diseases (Ahn *et al.*, 1994 and 1998).

The microbial flora, together with their interaction with host cells of the mucosa and the intestinal immune system, build a complicated intestinal ecosystem. The human gastrointestinal tract has a complicated "cross-talk" between the epithelial lining, the mucosal immune system and the luminal flora. The immune system of the gastrointestinal mucosa has distinct structural and functional features in comparison to the systemic immune system, and represents in many respects a separate immunological entity (Marth and Zeitz, 1998; Onderdonk, 1998). Many factors, of exogenous and endogenous type, may influence this ecological equilibrium, such as nutritional factors, bacterial-related factors and host factors (Savage, 1986; Onderdonk, 1998). In the human large intestine, the composition and density of bacterial populations are influenced by external pressures such as available nutrients, pH, the short chain fatty acid concentration (which in turn influences the pH in the colon), bile acids together with host factors. The sum of all these control mechanisms serves to build a barrier to colonisation by these enteric pathogens. In contrast, pathologically, this highly complex and sensible equilibrium can easily be disturbed by bacterial and viral infections, which may lead to bacterial overgrowth or to impoverishment of the normal gastrointestinal microflora (Onderdonk, 1998). As consequence, the following pathological processes may arise and will be discussed here.

The "bacterial translocation" eludes to the passage of particles or microorganisms from the gastrointestinal tract across the intestinal mucosal barrier which then appear in extraintestinal sites such as the lymph, mesenteric lymph node complex, liver, spleen, peritoneal cavity, and blood stream. When the ecological equilibrium is disrupted as indicated by a bacterial translocation, it may be caused by three major mechanisms: a heightened bacterial overgrowth, increased intestinal permeability or damage to the intestinal mucosa, and an impaired host immune defence (Berg, 1998). A bacterial translocation has been found e.g. in diabetes. Furthermore, experimental models revealed an increased intestinal permeability prior to the onset of this metabolic disease (Berg, 1998; Meddings *et al.*, 2003). In addition, the probability of bacterial translocation depends on the bacterial species. So, it was found that facultatively anaerobic bacteria translocated faster than the obligately anaerobic bacteria. A bacterial translocation may be the culprit in life-threatening opportunistic infections of immunosuppressed patients as well as the case of lethal sepsis (Berg, 1998).

Generally, the mucosal epithelial layer may be penetrated either by the transcellular (intracellular) or by the paracellular (extracellular) route. Different luminal influences are capable of influencing the rate and size when crossing these lines (Meddings *et al.*, 2003). In animal models, in which the intestinal epithelium is not physically damaged, the route of translocation of indigenous bacteria from the GI tract is through the intestinal epithelial cells (i.e. an intracellular route) rather than between the enterocytes via the tight junctions (i.e. an extracellular route). If the epithelial cells' tight junctions or intestinal epithelium are physically damaged, indigenous bacteria are then able to pass directly through the denuded or ulcerated areas to the lamina propria. Using the intracellular route, translocating indigenous bacteria use the lymphatic route to reach extraintestinal sites. In the shock models exhibiting physical damage to the mucosal barrier, translocating indigenous bacteria can travel by a vasculature route directly to the liver, in addition to the usual lymphatic route (Berg, 1998).

This intestinal permeability translates to the passive permeation of normally not absorbable molecules, macromolecules and bacterial toxins. This condition may be caused by mucosal lesions and ulcers. An increased mucosal permeability, or "a leaky gut" is made responsible for systemic endotoxinaemia, and endotoxin shock with multiple organ failure (Gardiner, 1998; Gabe *et al.*, 1998; Di Leo *et al.*, 2003).

In view of the necropsy reports concerning the sifakas, and in view of the pathologic processes and factors discussed above, it can be presumed, that a history of diarrhoea in the sifakas resulted in a damaged mucosal barrier and a weakened immune system. Both favoured the emergence of various systemic infections such as pneumonia, the development of multifocal infections, and finally, a septicaemia with a multiple organ failure. Both, the lymphatic and the blood system seemed to be implicated in this diagnosis. Exceptionally opportunistic bacteria and a presumed "leaky gut", can easily be used as port of entry into the animal host, which increases the risk of severe opportunistic infections. This theory is corroborated by the fact, that for many sifakas, the veterinarian failed to pinpoint the definite reason for the clinical course of their disease, and they died despite medicinal treatment.

In captivity, the animals became sick and died, albeit with no obvious detrimental or noxious food having been ingested. Therefore, the self-selected leaf species are concluded to exert predominantly diarrhoea-preventive properties onto the gastrointestinal milieu.

2 Materials and Methods

2.1 Assays on antimicrobial activity

The following *in vitro* assays serve to screen for possible antibacterial, antiprotozoal and antifungal properties within the sifakas' food plants. The main focus of these assays were performed on the leaves and leaf extracts of *R. copallina* and *A. julibrissin*.

Preliminary antibacterial assays

According to method A, only *R. copallina* and *A. julibrissin* leaves were investigated for possible antibacterial properties. According to method B, four additional leaf species of the browse, *Robinia pseudoacacia*, *Cercis canadensis*, *Liquidambar styraciflua*, *Acer rubrum*, were included. (All leaf species were harvested in May.)

Test strains ("wild type")
Salmonella Edinburgh
Staphylococcus aureus
Pseudomonas aeruginosa

Reference compounds and solutions
Gentamycin test disks (30 µg) (Oxoid);
Tannic acid (tannin, Fluka), utilised as solid compound and as 0.2 % (m/v) methanolic solution;
Gallic acid (Roth), utilised as solid compound and as 0.1 % (m/v) methanolic solution.

Method A
Methanolic leaf extracts: 1 g of powdered *R. copallina* and *A. julibrissin* leaves were extracted with 3 ml methanol, respectively.

Dry extracts of *R. copallina* and *A. julibrissin* leaves: approximately 1 g of the powdered leaf material (air-dried leaves) were weighed exactly and stirred in 30 ml methanol for 30 minutes at room temperature. The extracts were evaporated (Rotavapor®) and dried overnight at 70 °C in an oven. (*A. julibrissin* leaves yielded 13.5 % dry extract and *R. copallina* leaves yielded 55 % dry extract.) The dry extracts were tested as such.

Tannins extracted from *R. copallina* leaves (C.IV.2.5) were tested as such and as methanolic solution (0.1 % m/v).

Powdered leaf material of *R. copallina* and *A. julibrissin* leaves was tested as such.

The tests were conducted on agar dishes (Müller-Hinden-agar). Liquid extracts and solutions (the solved dry extracts and the solved reference compounds) were tested on paper disks (paper disk test, DAB 9). The dry extracts, the powdered leaf material, and solid compounds were directly put onto dishes. The solvent methanol was tested as blind. Commercial gallic and tannic acid (reference compounds) were tested as solid compounds and as methanolic solutions. Gentamycin paper disks were used as an antibiotic reference substance for each disc test. The paper disks were soaked several times with the prepared solutions, so that no final concentration of the loaded disks was determined. The incubation time lasted for 24 hours at 37 °C, respectively.

Method B
Approximately 30 mg powdered leaf material were weighed exactly, dispersed in 0.5 ml methanol and left to stand overnight. The suspension was centrifuged and 50 µl were taken respectively for each disk diffusion test. Methanol was tested as blind and gentamycin disks were utilised as reference compound.

The test procedure was adopted from method A. However, based on results from method A, only *Staphylococcus aureus* and *Pseudomonas aeruginosa* were utilised as test strains.

Screen on *R. copallina* and *A. julibrissin* leaves for antibacterial, antiprotozoal and antifungal properties

Assays were conducted by Solvay Arzneimittel GmbH, Germany, in cooperation with Panlabs (Inc.):

Approximately 4 g of the dried leaf material of *R. copallina* and *A. julibrissin* (May harvest) were finely ground and extracted twice with 50 ml ethylacetate overnight at room temperature. The supernatant solution was reduced *in vacuo*. The residue was re-extracted with 50 ml methanol overnight, respectively. Both extracts were unified, taken to dryness *in vacuo* and dried for several hours at 40 °C. The test compounds were dissolved in DMSO 0.15 % + absolute alcohol 0.3 % in aqueous media.

2.2 Cholera toxin binding assay

The cholera toxin binding assay was performed according to the method adopted from Hör *et al.* (1995). The toxin (0.16 µg / µl final concentration) was dissolved in a binding buffer (100 mM Tris, pH 6.8, 10 % (v/v) glycerol, 260 mM mercaptoethanol) and incubated for a few minutes with the various test samples (final concentrations varying from 30 ng / µl to 15 µg / µl) at room temperature. The mixture was heated for 5 minutes at 97 °C. The denatured proteins were analysed by SDS-PAGE[1] according to Laemmli (1970). The separating gel contained 14 % (m/v) acrylamide and 0.8 % (m/v) bisacrylamide. Gels were stained as described in the “Plus One Silver Staining Kit, Protein” from Pharmacia. Molecular weights were determined using the prestained SDS-PAGE standard (broad range) from Bio-RAD (approx. 95 % SDS-PAGE).

The assay was used to test tannin extracts from *R. copallina* leaves that were harvested in May, July and October. Leaf extractions were performed according to C.IV.2.5. Furthermore, p- and m-digallic acid methyl ester (p- and m-methyl digallate) were tested, which had been isolated as mixture in fraction R16 by preparative HPLC method (App. II.3, No.2). Commercial tannic acid (Fluka) and tannin albuminate (Knoll Deutschland GmbH / BASF Pharma, now Dr. Rentschler Arzneimittel GmbH) were tested as reference compounds. Cholera toxin was purchased from Sigma [9012-63-9]. For the majority of samples, duplicate assays were performed.

Comment:
Gelelectrophoresis separates proteins into different fractions according to their mass, electric charge and tertiary / quaternary structure. In the presence of SDS, proteins are completely unfolded and the electric charge is masked by the anionic detergent binding to the proteins. Accordingly, the electrophoretic mobility of a given protein is exclusively dependent on its mass. Under mild conditions, however, the interaction of proteins with certain secondary metabolites is not completely abolished, and the study of such interactions using SDS-PAGE is possible.

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[1] Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

2.3 Calculation of the daily intake of flavonoids and tannins

To evaluate the significance of the secondary plant compounds ingested by the sifakas, the contents of the flavonoids and tannins were determined from the air-dried plant material (chapter C.IV, Tab. C.13, p. 119, to determine gallotannins and flavonoid glycosides, and Tab. C.16, p. 125, to determine flavonoid aglyca) and were calculated by means of the following formula:

$$\text{amount}_{\text{ingested}} = \frac{\sum^{\text{leaf species}} \left(c_{\text{fresh}} \times \sum^{\text{days}} \text{weight}_{\text{eaten}} \right)}{t}$$

$\text{amount}_{\text{ingested}}$	the averaged daily amounts [g] of the flavonoids and tannins, respectively ingested by each lemur group;
c_{fresh}	the content (m/m) of the flavonoids and tannins referred to the fresh leaf species;
$\text{weight}_{\text{eaten}}$	the weight [g] of the fresh leaf species eaten by each respective lemur group;
t	the number of days of each group's observation period.
$\text{intake}_{\text{per bodyweight}}$	the averaged daily amounts of the flavonoids and tannins, respectively ingested by each lemur group ($\text{amount}_{\text{ingested}}$) and divided by each group's total bodyweight [g / kg];
$\text{intake}_{\text{per individual}}$	the averaged daily amount of the flavonoids and tannins ingested per bodyweight ($\text{amount}_{\text{per bodyweight}}$) and multiplied by the bodyweight of each individual lemur of the respective animal group [g].

In this calculation, all browse species, plus the respective amounts ingested within the forested enclosure (Tab. B.9, p. 43) were included. The calculations were performed separately for each observation period, i.e. the spring and autumn. For each animal's bodyweight see chapter B, Tab. B.1, p. 17.

3 Results

3.1 Antimicrobial activity

Preliminary studies

During a series of preliminary assays, various leaf extracts from *R. copallina* and *A. julibrissin* were investigated for their growth-inhibitory activities against three bacterial strains, *Staphylococcus aureus*, *Salmonella* Edinburgh and *Pseudomonas aeruginosa* ("wild types"), (Tab. C.20). As gallic acid may be hydrolysed from gallotannins during the gut passage, gallic acid was included in these first assays. Commercial tannic acid, gallic acid and gentamycin were used as reference compounds (method 2.1).

As depicted in Tab. C.20, the methanolic leaf extract and the tannin extract (C.IV.2.5) from *R. copallina* leaves showed growth-inhibitory activities against *St. aureus* and *Ps. aeruginosa*. No activity could be found against *Salmonella* E., so it was not utilised in further test series. The same activity pattern was found for methanolic solutions of commercial tannic and gallic acid.

Tab. C.20: Growth-inhibitory activity of *A. julibrissin* and *R. copallina* leaf preparations against three bacterial strains ("wild types").

test samples	***St. aureus***	***Ps. aeruginosa***	***Salmonella* E.**
A. julibrissin leaves[a]	–	–	–
R. copallina leaves[a]	+	+	–
R. copallina tannin extract[b]	+	+	–
tannic acid (Fluka)[c]	+	+	–
gallic acid (Roth)[c]	+	+	–
gentamycin[d]	+	+	+

a Powdered leaf material, and dry and methanolic leaf extracts were assayed.

b The tannin extract from *R. copallina* leaves (C.IV.2.5) was tested as dry extract and as methanolic solution.

c Gallic and tannic acid were tested as solid compounds and methanolic solutions.

d gentamycin test disks (30 µg) (Oxoid)

— no inhibition zone visible

\+ inhibition zone visible

Solid samples as in the dried leaf extract and the dry tannin extract from *R. copallina* leaves, as well as powdered *R. copallina* leaves, showed the same inhibitory tendencies, but were less active than the methanolic extracts, especially against *Ps. aeruginosa*. This outcome was also found in solid tannic and gallic acid, whereas the latter produced small holes in aspects of the agar surface.

A. julibrissin leaf extracts showed no activity. Gentamycin as a standard showed nearly the same activity against all three bacterial strains. These results indicate an antibacterial activity for *R. copallina* leaves that can be attributed to their content of gallotannins.

To compare antibacterial activities between the different food plants of the browse fed in May, assays also included the other four leaf species of the browse (Tab. C.21).

Tab. C.21: Antimicrobial assays including all food species of the browse offered in May.

		inhibitory zone	
test samples	**content (disc^{-1})**	***St. aureus***	***Ps. aeruginosa***
Rhus copallina	3 mg	2 mm	3 - 4 mm
Acer rubrum	3 mg	-	2 mm
Cercis canadensis	3 mg	1 mm	-
Liquidambar styraciflua	3 mg	-	-
Albizia julibrissin	3 mg	-	-
Robinia pseudoacacia	3 mg	-	-
methanol	50 µl	-	-
gentamycin	30 µg	9 - 11 mm	11 mm

While *A. julibrissin*, *R. pseudoacacia* and *L. styraciflua* tested negative against either bacterial strain, *C. canadensis* scored a slightly positive finding against *St. aureus*, and *A. rubrum* against *Ps. aeruginosa*. *R. copallina* leaf extracts showed the highest activity against both bacterial strains (Tab. C.21).

Although *A. rubrum* leaves were also found to contain large amounts of hydrolysable tannins (see chapter C.IV), they were less effective in this assay than *R. copallina* leaves.

3.2 Antibacterial, antiprotozoal and antifungal properties of *R. copallina* and *A. julibrissin* leaf extracts

In a detailed serial examination conducted by Panlabs Inc., the most preferred leaf species by the sifakas, *R. copallina* and *A. julibrissin*, were tested for possible antimicrobial activities against different strains of gram positive, gram negative and anaerobe bacteria, one protozoon and several fungal species (Tab. C.22).

In agreement with the aforementioned assays, only plant extracts from *R. copallina* leaves revealed any antimicrobial activity, while there was no observed effects for *A. julibrissin* leaf extracts. Therefore, only effects for *R. copallina* leaf extracts were presented in Tab. C.22.

Tab. C.22: *In vitro* tests on potential antibacterial properties of *R. copallina* leaf extracts. Gentamycin was used as reference compound.

bacteria	minimal inhibitory concentration (µg / ml) *R. copallina*	gentamycin
gram positive bacteria		
Bacillus subtilis (ATCC 43223)	> 100	0.03
Corynebacterium minutissimum (ATCC 23347)	100	0.1
Mycobacterium ranae (ATCC 110)	> 100	0.3
Staphylococcus aureus (ATCC 6538 P)	100	0.1
Staphylococcus aureus, methycillin resistant (ATCC 33591)	100	1
Staphylococcus aureus (Smith)	100	0.1
Staphylococcus aureus (Smith + 50 % calf serum)	> 100	0.03
Staphylococcus faecalis (ATCC 10541)	> 100	30
gram negative bacteria		
Escherichia coli (ATCC 10536)	> 100	0.3
Escherichia coli (Juhl)	> 100	0.3
Klebsiella pneumoniae (A 9977)	> 100	0.1
Proteus vulgaris (A 9539)	> 100	0.3
Pseudomonas aeruginosa (ATCC 9027)	30	0.3
anaerobe bacteria		
Bacteroides fragilis (ATCC 23745)	100	30
Clostridium sporogenes (ATCC 7955)	> 100	0.1
Corynebacterium acnes (ATCC 6919)	30	1
Helicobacter pylori (ATCC 43540)	100	0.3

The minimal relevant inhibitory concentration was set at 100 µg / ml throughout these serial examinations.

From the bacterial strains tested (Tab. C.22), growth-inhibitory effects could be evidenced for *R. copallina* leaf extracts at 100 µg / ml against *Corynebacterium minutissimum*, several *Staphylococcus* strains, *Bacteroides fragilis* and *Helicobacter pylori*. A minimal concentration of 30 µg / ml was sufficient to inhibit *Pseudomonas aeruginosa* and *Corynebacterium acnes* in growth.

During the search for possible antiprotozoal properties, *Trichomonas foetus* (Br. M. Strain) was tested. Metronidazole was used as reference compound. At a minimal inhibitory concentration of 100 µg / ml, neither *R. copallina*, nor *A. julibrissin* leaf compounds exhibited any observable activity.

To screen for possible antifungal properties, a large range of different fungal species were assayed, but all tested negative by both plant species. Hence, only test species assayed were listed in the following table (Tab. C.23). Amphotericin B was used as reference substance for this serial examination.

Tab. C.23: Species of fungi, which were tested negative against leaf extracts of *R. copallina* and *A. julibrissin*.

mammalian pathogens	
Candida albicans (ATCC 10231) *Exophilia jeanselmei* (ATCC 10224) *Microsporum gypseum* (ATCC 14683) *Trichophyton rubrum* (ATCC 10218)	*Epidermophyton flocosum* (ATCC 18397) *Microsporum canis* (ATCC 36299) *Trichophyton mentagrophytes* (CDC Atlanta)
general	
Aspergillus niger (FA 9959) *Mucor hiemalis* (ATCC 8977a) *Penicillium chrysogenum* (ATCC 9480) *Saccharomyces cerevisiae* (ATCC 9763)	*Aspergillus niger* (FA 24199) *Paecilomyces varioti* (ATCC 22319) *Pitysporum ovale* (ATCC 38593)
plant pathogens	
Alternaria alternata (foliar) (ATCC 13963)	*Cochliobolus heterostrophus* (foliar) (ATCC 11534)
Fusarium moniliforme (root) (CCRC 30972)	*Phytophthora capsici* (foliar) (ATCC 15399)
Rhizopus stolonifera (root) (ATCC 24862)	*Trichoderma viride* (wood) (CCRC 30993)
materials degradation, spoilage	
Aureobasidium pullulans (paint) (ATCC 9348)	*Stachybotrys chartarum* (wool, cotton) (ATCC 16026)

In summary, *R. copallina* leaf extracts showed dose-dependent antibacterial effects. *Acer rubrum* and *Cercis canadensis* were the only other leaf species of the browse that tested positive during the preliminary assays, but were less active than *R. copallina* leaf extracts and therefore, were not investigated further.

In this study, *R. copallina* leaf extracts exhibited antibacterial activities against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Corynebacterium minutissimum*, *C. acnes*, *Bacteroides fragilis* and *Helicobacter pylori* (Tab. C.22). On the contrary, neither antiprotozoal, nor antifungal properties could be found against the strains assayed. *A. julibrissin* leaf extracts showed no activity.

Based on these preliminary assays, it may be concluded that the antibacterial activity originates primarily from the content of hydrolysable tannins within the *R. copallina* leaves. In this *in vitro* assay, gallic acid also showed antibacterial properties. Gallic acid is hypothesised to contribute to a limited extent to the antibacterial effect of the hydrolysable tannins while being released through the gut passage (Tab. C.20). However, this assumed intensification and prolongation of antibacterial effects by gallic acid needs verification under *in vivo* conditions.

3.3 Binding capacity of various *R. copallina* leaf extracts to cholera toxin

In the following assay, the cholera exotoxin produced by the bacteria *Vibrio cholerae* was used to characterise possible antidiarrhoeal properties of the tannins extracted from *R. copallina* leaves. The binding capacity to cholera toxin was screened *in vitro* by gel-electrophoresis. This assay has been adopted from Hör *et al.* (1995), who had screened proanthocyanidins from *Guazuma ulmifolia*, a plant which is used as antidiarrhoeal by indigenous people of Mexico. It should be noted that the enterotoxin of pathogenic *E. coli* strains, are often the cause of traveller's diarrhoea, and is similar to the cholera toxin in both structure and effect (Rimpler, 1999). As tannins of the bark of *Guazuma ulmifolia* inactivated the cholera toxin (Hör *et al.*, 1995), a similar effect was hypothesised for the gallotannins utilised against traveller's diarrhoea (Rimpler, 1999). According to Thorne (1986), *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) are the prime examples of a bacterial group that attach to the mucosa in the proximal small bowel and produce a potent enterotoxin(s) that causes fluid secretion but does not destroy the brush border of the intestinal villi.

Cholera toxin is an 87 kDa protein produced by *Vibrio cholerae* consisting of 5 B subunits of 12 kDa each and one A subunit of 27 kDa, the latter being further divided into A_1 (22 kDa) and A_2 (5 kDa) subunits. The B subunits form a planar pentagonal ring surrounding the A subunit (Voet and Voet, 1990; Hör *et al.*, 1995).

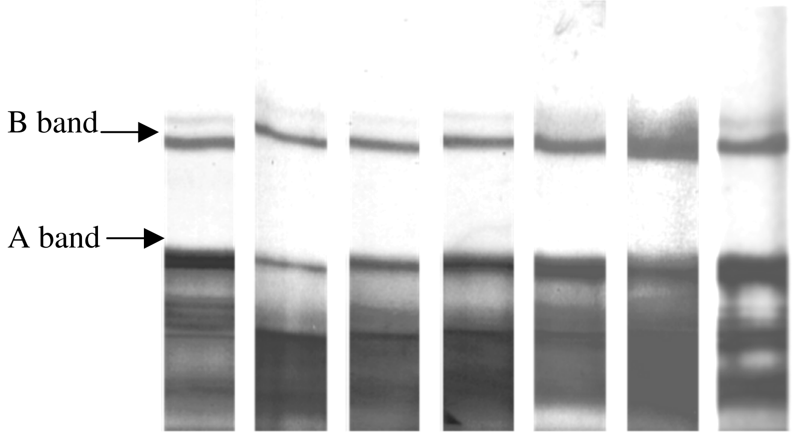
The B subunits bind the toxin to receptors (GM_1 ganglioside) present on the surface of mucosal enterocytes. As the B subunits bind to GM_1 receptors, a conformational change is believed to occur by which the A_1 subunit gains entry into the enterocyte (receptor-mediated endocytosis), while the B subunits do not penetrate the cell (Thorne, 1986). This process triggers cholera toxin activation by proteolytic cleavage and disulfide bond reduction of the A subunit leading to two fragments, A_1 (22 kD) and A_2 (5 kD).

In the following, the A_1 subunit catalyses activation of an adenylate cyclase, which is locked in its active state causing a giant increase in intracellular cAMP concentration, which in turn induces the secretion of enormous quantities of digestive fluid, low in protein and rich in electrolytes (Thorne, 1986; Voet and Voet, 1990).Thus, the toxin stimulates chloride secretion by activating the cAMP second messenger system (Hör *et al.*, 1995).

The binding effect could best be observed for *R. copallina* tannin extracts (gained according to C.IV.2.5) in concentrations varying from 30 ng / µl to 300 ng / µl (see Fig. C.13).

Higher concentrations (1.5 µg / µl and 15 µg / µl) were stained by the silver staining procedure which made the detection of proteins difficult.

Highest affinity to the enterotoxin was exhibited by the tannin extract gained from *R. copallina* leaves harvested in May. 30 ng / µl already yielded a considerable decrease in the A subunit band (Fig. C.13, lane 2) if compared to the control lane of mere cholera toxin (lane 1). In this assay, the decrease in the A subunit band was somewhat less pronounced using the same concentration of tannin extract gained from *R. copallina* leaves of July. In contrast, 150 ng / µl were needed of the tannin extract from October leaves to achieve approximately the same decrease in A band as shown for 30 ng / µl of the July leaf extract (lane 3 and 4).



7 Tannin albuminate 60 ng / µl
6 Tannic acid 1.5 µg / µl
5 R 16, 1.5 µg / µl
4 Tannin extract from October leaves, 150 ng / µl
3 Tannin extract from July leaves, 30 ng / µl
2 Tannin extract from May leaves, 30 ng / µl
1 Cholera toxin without tannin sample (control)

Fig. C.13: Binding of the cholera toxin by tannin extracts gained from *R. copallina* leaves of May, July and October including the isolated fraction R16 in comparison to commercial tannic acid and tannin albuminate examined by SDS-PAGE. The final concentration of cholera toxin is constant for all lanes (0.16 µg / µl).

In lane 5 (Fig. C.13) the binding capacity of fraction R16 containing p- and m-digallic acid methyl ester is shown. To observe any binding effect, at least 1.5 µg / µl were required of this fraction. Similarly, high concentrations of commercial tannic acid were needed to state a first decrease in the A band (1.5 µg / µl, lane 6).

As tannin albuminate is a 1:1 tannin to protein complex, twice the concentration was used when compared to other tannin extracts. A slight decrease in the A band could already be seen using 2 x 30 ng / µl. However, this effect was considerably less pronounced when compared to the various tannin extracts from *R. copallina* leaves (Fig. C.13, lane 7 versus 2, 3, 4).

The binding of tannins extracted from October leaves to the cholera enterotoxin in concentrations of 30, 150 and 300 ng / µl and the gradual disappearance of the A subunit band is depicted in Fig. C.14. Again, using concentrations higher than 300 ng / µl lead to strong staining of the gel and the disappearance of the protein bands. The corresponding lanes are therefore not depicted here.

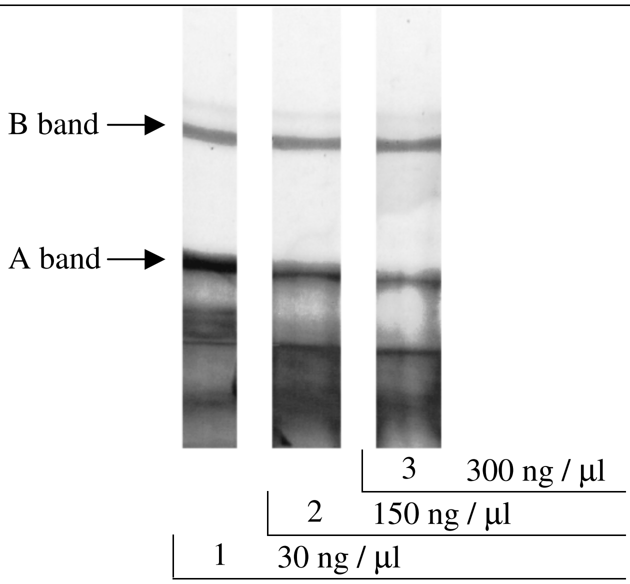


Fig. C.14: Binding of the cholera toxin by tannins extracted from *Rhus copallina* leaves harvested in October. The final concentration of cholera toxin is constant for all lanes (0.16 µg / µl).

In agreement with results of Hör *et al.* (1995), in this *in vitro* assay, the tannin samples gained from *R. copallina* leaves of different seasons have bound to the enzymatically active A subunit of the cholera exotoxin causing a dose-dependent decrease of this band while the B subunit has been largely unaffected. Tannin extracts from *R. copallina* leaves exhibited better binding properties to the A subunit of the cholera toxin than either commercial tannic acid or tannin albuminate. The mixture of purified p- and m-digallic acid methyl ester (fraction R16) was not as effective as the entire leaf tannin extracts. It is reasonable to conclude that seasonal differences in the composition of *R. copallina* leaf extracts are responsible for the different affinities in binding to the toxin. It may be concluded that there is a slight difference in the binding capacity between May and July leaf extracts with a minor enhancement with May leaves. Both origins were superior to leaves harvested in October. However, the determination of exact quantitative binding activities between *R. copallina* leaf tannin extracts from different seasons needs further investigation.

3.4 Potential biological and pharmacological properties of the major food plant chemicals: evidence from the literature and own studies

Flavonoids

Flavonoids constitute the most abundant group of secondary compounds in plant kingdom to which a large range of ecological, biological and pharmacological effects has been attributed, such as anti-haemorrhagic, anti-oedematous, anti-inflammatory, antimicrobial, spasmolytic, diuretic, hepatoprotective, cardiovascular, antioxidative, radical-scavenging, and enzyme inhibiting as well as enzyme-inducing properties (Barz and Hösel, 1975; Harborne, 1979; Steinegger and Hänsel, 1988; Harborne and Grayer, 1994; Middleton and Kandaswami, 1994; Beil *et al.*, 1995; Harborne, 1999; Wagner, 1999; Cos *et al.*, 2001; Nijveldt *et al.*, 2001).

For quercitrin, afzelin, and luteolin, that have been isolated from *A. julibrissin* and / or *R. copallina* leaves in this study (Tab. C.8, p. 107), a radical scavenging activity has been reported (Jang *et al.*, 2002; Jung *et al.*, 2004a). In addition, for quercetin it was found that it suppressed cytotoxicity and prevented cell damage in mammalian and bacterial cells (Nakayama, 1997; Le Marchand, 2002).

Concerning diarrhoeal diseases, beneficial effects to the gastrointestinal tract have been found for quercetin glycosides such as quercitrin (Gálvez *et al.*, 1993a, b; Gálvez *et al.*, 1995; Rao *et al.*, 1997), which was found to be the most prevalent flavonoid glycoside in the sifakas' food plants in this study. Remarkably, flavonoids were vital when it came to intestinal repair and had a dramatic impact on gut motility and fluid and electrolyte secretion in a dose-dependent manner, which depended upon the flavonoid structure (Di Carlo *et al.*, 1993), and the diarrhoea provoking agent (Di Carlo *et al.*, 1993; Gálvez *et al.*, 1993a, b; 1995; Rao *et al.*, 1997).

However, no antidiarrhoeal medicine, that explicitly contains flavonoids, has ever been introduced into human medicine as of late (Rote Liste, 2005).

Under experimental conditions, quercitrin was found to delay the transit time of the small intestine in rats, but only if it had been accelerated by secretagogue agents (e.g. castor oil, prostaglandin E_2, sodium picosulfate). Quercitrin was neither active under normal conditions, nor in models, which were not associated with modifications in electrolyte and water transport, such as osmotic diarrhoea induced by magnesium sulphate (Gálvez *et al.*, 1993a, b). In models with changed fluid transport, quercitrin increased the electrolyte and fluid absorption by the intestinal and colonic mucosa. While the stool weight and the number of faeces were reduced by quercitrin in castor oil induced diarrhoea, only the stool weight decreased in the prostaglandin model (Gálvez *et al.*, 1993a).

In lactose induced chronic diarrhoea, rats treated with quercitrin recovered quicker from diarrhoea than control animals. The treated rats had a lower number of faecal output and their gain in body weight was significantly greater compared to rats who did not receive quercitrin after removal of lactose from the diet. Furthermore, quercitrin effectively helped to restore the mucosal weight to normal values, which had been increased during diarrhoea. Mucus secretion is an important protective measure of the colon, which is enhanced whenever the mucosa is irritated (Gálvez *et al.*, 1995).

In other models, different flavonoids have been found to influence the intestinal transit time and gut motility e.g. by decreasing the amplitude of phasic contractions and to antagonise contractions induced by agents such as prostaglandins, acetylcholine, histamine and serotonin. For instance, quercetin, inhibited anaphylactic contraction of guinea pig ileum in a concentration-dependent manner (Fanning *et al.*, 1983; Lutterodt, 1989; Meli *et al.*, 1990; Capasso *et al.*, 1991; Di Carlo *et al.*, 1993; Middleton and Kandaswami, 1994; Rao *et al.*, 1997). Some flavonoids were able to prevent gastric mucosal damage to a limited extent in different test models when administered in advance (Rao *et al.*, 1997).

One important mechanism of flavonoids (including quercetin) is their manifold interference with calcium-dependent processes, which may be responsible for their observed spasmolytic mode of action on gastrointestinal functions (Fanning *et al.*, 1983; Di Carlo *et al.*, 1993; Middleton and Kandaswami, 1994). For example, kaempferol, myricetin, apigenin, and rutin delayed the transit times in the small and the large intestine in mice and rats and reduced (in a dose-dependent manner) the intestinal fluid and electrolyte secretion. All these effects could be enhanced by verapamil, a calcium-channel blocker (Di Carlo *et al.*, 1993).

Another important mechanism in the context of diarrhoea may be the interference of flavonoids with the arachidonic acid metabolism (Middleton and Kandaswami, 1994; Nijveldt *et al.*, 2001; Dingermann and Loew, 2003; Van Wyk and Wink, 2004). Both the mucosa and muscle layers of the gut possess the ability to generate all major products of the arachidonic acid metabolism including prostaglandins, which are known to play an important role in diarrhoeal diseases. Many flavonoids such as quercetin and rutin are revealed to be inhibitors of the cyclooxygenase and lipoxygenase enzyme (Gálvez *et al.*, 1993a; Cos *et al.*, 2001; Dingermann and Loew, 2003). However, an prostaglandin-independent mechanism has also been proposed (Gálvez *et al.*, 1993a; Beil *et al.*, 1995).

With respect to the necropsy reports it is interesting to note that some flavones which have beneficial effects against periodontal diseases have also been described as having anti-inflammatory and tissue regenerating effects (Chung *et al.*, 1995).

In many assays, quercetin exhibited the most potent properties (Di Carlo *et al.*, 1993; Middleton and Kandaswami, 1994) and appeared to be the active principle of quercitrin and other quercetin glycosides, which needed to be metabolised by colonic bacteria in advance (Lutterodt, 1989; Gálvez *et al.*, 1993a; 1995). Hence, some important notes on the knowledge of pharmacokinetic processes of flavonoids will be presented.

Data on the pharmacokinetic of flavonoids are scarce and sometimes contradictory, but highly relevant in assessing their beneficial effects on human and animal health. The rate and extent of intestinal absorption and the nature of the metabolites circulating in the plasma seem to depend on their chemical structure such as hydroxylation and glycosylation, and the type of food source. Since an extensive metabolism is in evidence, the species- and tissue-specific enzymatic equipment of an animal host (depending e.g. on its development) and its microflora (depending e.g. on its population density and diversity) are crucial. Genetic polymorphisms of enzymes, enzymatic specificity, activity, inducibility, and distribution are of great importance. This helps to explain the enormous difference in interspecies and interindividual bioavailability of (poly)phenolic plant compounds (Hollman and Katan, 1997; Scalbert and Williamson, 2000).

The first step in the metabolism of flavonoids is assumed to be the enzymatic cleavage of the sugar moieties yielding free aglyca. This deconjugation step is performed by intestinal enzymes from the animal host or those secreted by the gut microflora, especially of the colon. Subsequently, the free aglyca are (re)conjugated, either by intestinal enzymes again and / or later in the liver and kidneys yielding methylated, sulphated and / or glucuronidated derivatives. Depending on the applied dosage, free aglyca may also diffuse across the gut wall to enter the blood stream, or may be further degraded by the gut microflora by ring fission producing simple phenolics, which can then be easily absorbed. As metabolic pathways may be dose-dependently saturated, unconjugated free aglyca are more likely to be found in the plasma after the application of high (pharmacological) doses than after low (nutritional) doses. The primary site of metabolism also appears to be dose-dependent, in that the liver plays a subsidiary role to the intestine in the metabolism of small doses, but is more important if large doses are administered. Therefore, the small intestine-liver pathway is the crucial step in the first-pass metabolism of flavonoids (Scalbert and Williamson, 2000). Overall, it seems unlikely that unmetabolised flavonoid glycosides are absorbed through the gut wall (Hollman and Katan, 1997; Scalbert and Williamson, 2000; Day and Williamson, 2001; Sesink *et al.*, 2001; Day and Williamson, 2001; Németh *et al.*, 2003).

While in humans e.g. glucosides are potential substrates for endogenous ß-glucosidases expressed by the human enterocytes, rhamnosides need α-rhamnosidases produced by the colonic microflora. This helps to explain the differences in human plasmatic maximum concentrations of metabolites as generated by enterocytes (most often 1-2 h after ingestion of flavonoids) and of metabolites after partial degradation by the colonic microflora (e.g. 9 h for rutin after ingestion). Nonenzymatic declycosylation, such as in the acidic pH of the stomach, does not occur in the human body (Scalbert and Williamson, 2000; Day and Williamson, 2001; Németh *et al.*, 2003).

Flavonoids are predominantly eliminated by biliary excretion, and typically only trace amounts are found in the urine (Hollman and Katan, 1997; Scalbert and Williamson, 2000; Day and Williamson, 2001; Németh *et al.*, 2003). The main metabolites of quercetin and rutin were hydroxylated and methoxylated phenyl acetic acids, which were found in the urine of guineapigs, rabbits, rats and humans (Dingermann and Loew, 2003). Overall, the polyphenol concentration in the gut should be much higher than in the plasma (Scalbert and Williamson, 2000).

As predominantly rhamnose containing flavonoid glycosides were evidenced to occur in the major food plants of the focal sifakas (Tab. C.8, p. 107), an extensive metabolism by the gut microflora can be expected. Beneficial effects are assumed to be confined primarily to the gut. Only to a minor extent are systemic effects to be expected due to a low rate of absorption and an extensive microbial metabolism.

So, it seems reasonable to conclude that flavonoids, especially with quercetin as aglycone like quercitrin, contribute significantly to the repair and restoration of the gastrointestinal mucosa after being injured from pathological episodes such as acute or chronic diarrhoea.

Tannins

The tannin group can be divided into condensed tannins (proanthocyanidins; catechin or leucocyanidin units) and the hydrolysable tannins (gallotannins and ellagitannins) (Rimpler, 1999). While condensed tannins reach molecular weights of up to 20 000 Da, hydrolysable tannins exhibit molecular weights of up to 3000 Da.

Tannins can be characterised as polyphenolic compounds with the ability to complex with alkaloids, multiple charged cations, gelatine, and - most importantly in ecological and pharmacological implications - polysaccharides and proteins. The original meaning of "tannin" is the ability to precipitate hides (collagen of the skin), that is to produce leather, which is based on irreversible covalent bonds between tannins and proteins. The astringent potency - noticed by a harsh, astringent taste - is due to reversible noncovalent bonds (like hydrogen bonding and even more important hydrophobic associations[1]) between tannins and glycoproteins of the oral cavity and the gastrointestinal tract (Bate-Smith, 1977; Haslam, 1988; Hagerman, 1992; Baxter *et al.*, 1997). However, the astringent potency, which is much more relevant for pharmacological interactions, does not parallel the capacity of binding to hides (Steinegger and Hänsel,1988).

Maximum astringent properties are found for tannins of intermediate size with a molecular weight in the range of 500-3000 and 1-2 hydroxyl groups per 100 mass units (Haslam, 1975; 1988; Wagner, 1999).

Tannins constitute an ubiquitous group of plant compounds, which is known to possess a variety of ecological (Swain, 1979) and pharmacological activities (Steinegger and Hänsel, 1988; Rimpler, 1999; Rote Liste, 2005).

It should be noted that condensed tannins are processed differently from hydrolysable tannins within the digestive tract, hydrolysable tannins are subject to enzymatic hydrolysis (Hagerman, 1992; Butler and Rogler, 1992). Bate-Smith (1977) interpreted the hydrolysability and the superior astringent potency of hydrolysable tannins as providing an evolutionary advantage, (hydrolysable tannins are superior to condensed tannins and in turn gallotannins are superior to ellagitannins). The dominance of hydrolysable tannins in dicots compared to ferns and gymnosperms (Bate-Smith, 1977) and the clear predilection of the sifakas for hydrolysable tannins compared to the condensed type may be interpreted as following this "trend of evolution."

Internally, the prophylaxis and treatment of acute unspecific and chronic diarrhoeas, with and without emesis, constitute the classical field for the group of hydrolysable tannins (e.g. in Tannalbin®). Externally, drugs containing tannins are used as gargle, for infections concerning the throat and mouth, e.g. gingivitis, for caries prophylaxis, for wounds and skin lesions, itches, haemorrhages and haemorrhoids (Steinegger and Hänsel, 1988; Rimpler, 1999; Rote Liste, 2005).

[1] According to Haslam *et al.* (1989) and Hagerman (1992), ionic bondings are unlikely to be involved in the formation of tannin-protein complexes, as tannins do not interact with proteins at pH values above 10, indicating that the phenolate anion is not involved in complex formation.

In this study, tannin albuminate is used as a reference compound to discuss the effects of hydrolysable tannins (see C.VI.3.5). For tannin albuminate, astringent properties are evidenced by a significant protection of the intestinal mucosa, normalisation of hypersecretory processes, lowering of the gut passage rate and increased faecal consistency (scientific information on Tannalbin®). In the gut, the tannin moiety is continuously deliberated from the tannin-protein complex in neutral to alkaline pH conditions. Thus, the tannin moiety can develop its astringent properties proportional to its solubility throughout the entire gut until it reaches the lower parts, such as the colon. The colloidal structure of the mucosal surface including the capillaries are compressed, and the absorption of toxins is inhibited. In turn, toxin-induced secretion of water and electrolytes is also inhibited. Intestinal contents increase in consistency due to the precipitation and gelling processes of the involved proteins and glycoproteins (Schmid, 1952, Hoppe, 1995).

In early studies on tannin albuminate and tannic acid, a direct anti-inflammatory activity was found (Wallbach, 1939). Later, this was corroborated by findings on inhibition of lipoxygenase and prostaglandin H synthase by gallic acid esters *in vitro* and *in vivo* (Kroes *et al.*, 1992). This may in turn emphasise the efficacy of tannin albuminate within inflammatory bowel diseases (Plein *et al.*, 1993; Raedsch, 1995; Fiocchi, 1997). However, the mode of the tannin's actions can be characterised as a surface phenomenon (Haslam *et al.*, 1989).

While a systemic application of tannins is relatively toxic, orally administered tannins are scarcely absorbed by the gut. Orally administered tannin albuminate does not ensue detectable levels of tannic acid or gallic acid in the blood serum, and small amounts of tannic acid can be found even in faecal samples. This indicates a continuous deliberation and provision of gallotannin throughout the entire gut (scientific information on Tannalbin®). In contrast, after ingestion of pure tannic acid, no faecal excretion of intact tannic acid can be detected - an indication of its entire degradation within the upper (human) gastrointestinal tract (scientific information on Tannalbin®). Orally administered pure gallic acid rapidly appears as 4-O-methylgallic acid and unmetabolised gallic acid within human plasma and urine (Shahrzad and Bitsch, 1998).

In this study, faecal samples of the Coquerel's sifakas were analysed by HPLC technique (C.IV.2.1). These samples were found to contain gallic acid[1]. This finding may indicate the availability of gallotannins until food reaches the lower aspects of the gut with a "protracted effect" when continuously released from a tannin-protein complex. For the sifakas this would implicate a tannin-protein complex that may have formed e.g. by complexing with proteins from the saliva.

Further effects found in the hydrolysable type of tannins, appear to possess both antibacterial (Ahn *et al.* 1994; 1998) and antiviral (Kakiuchi *et al.*, 1985; Nakashima *et al.*, 1992; Hattori *et al.*, 1995; Kurokawa *et al.*, 1995; Büechi, 1998; Nakano *et al.*, 1998) properties.

To some extent gallic acid may be hydrolysed through the gut passage from the gallotannin and thereby able to exert additional or synergistic pharmacological effects. Thus, its antibacterial (Richards *et al.*, 1994; Kayser and Kolodziej, 1997; Kolodziej and Kayser, 1998; Ahn *et al.*, 1994; 1998) and anti-inflammatory properties (Liu *et al.*, 1983; Kroes *et al.*, 1992) may become important in this respect.

Moreover it should be mentioned that hydrolysable tannins (including their gallic acid monomer and derivatives such as gallic acid methyl ester and ellagic acid derivatives) are reported to possess antioxidant and radical scavenging (Hong *et al.*, 1995; Masaki *et al.*, 1994; 1997; Kim *et al.*, 2001), cytoprotective (Kanai, 1996; Kolodziej and Kayser, 1998) and tumor preventing properties (Kashiwada *et al.*, 1992; Perchellet *et al.*, 1992; Gali *et al.*, 1992;

[1] After the long storage of the faecal samples in simple test tubes until being analysed, no intact gallotannin was expected to be found.

Inoue *et al.*, 1994; Inoue *et al.*, 1995; Sakagami and Satoh, 1996; Sakagami *et al.*, 1997; Sakaguchi *et al.*, 1998).

However, many of the pharmacological and ecological effects of tannins strongly depend on their molecular size, their structure and stereochemistry (Haslam, 1988; Haslam *et al.*, 1989; Hagerman, 1992; Kashiwada *et al.*, 1992; Nakashima *et al.*, 1992) and the test system used (Hagerman, 1992). The complexing property of tannins produces manifold interactions. Therefore, the discovery of antinutritional and detrimental effects of tannins towards herbivorous species such as insects, chicks, rats, mice and some ruminants were the main reasons in the attribution of their "feeding deterrent" and "plant protective" abilities to the group of tannins in the evolutionary "arms race" with the predacious herbivores (Freeland and Janzen, 1974; Swain, 1979). The antinutritional effects described for diets high in tannins are known to comprise growth inhibition, diminished weight gains, lower efficiency in nutrient utilisation (particularly in protein) and increased levels of faecal nitrogen (Butler and Rogler, 1992; Baxter *et al.*, 1997).

Conversely, the adaptation to cope with tannins is a powerful force within an animal species (Baxter *et al.*, 1997), and this point is often ignored in the discussions concerning the benefits of tannins within ecology and pharmacology.

In this respect, the tannin-protein interaction is vital, occurring *in vivo* and *in vitro*. Tannins may complex with proteins of different origins such as dietary protein (here plant proteins), digestive enzymes, proteins of endogenous origin like salivary proteins, membrane bound proteins of the gastrointestinal mucosa, secretions, or microbial proteins and enzymes. These interactions are influenced by characteristics of the protein (e.g. molecular size, steric properties, composition, iso-electric point), by characteristics of the tannin (e.g. molecular size, structure, steric properties), and the reaction conditions (e.g. pH, temperature, time, solvent composition, concentration of both reactants), to form soluble or insoluble complexes by reversible, noncovalent bonds (Haslam, 1988; Haslam *et al.*, 1989; Hagerman, 1992; Baxter *et al.*, 1997). Thus, gallotannins exhibit a stronger astringent potency than the more rigid and compact formed ellagitannins (Haslam, 1988; Haslam *et al.*, 1989). Gallotannins in turn, particularly pentagalloylglucose, bind strongly to proteins which are rich in proline (Haslam, 1988; Haslam *et al.*, 1989; Butler and Rogler, 1992; Charlton *et al.*, 1996; Baxter *et al.*, 1997). In this context it is notable that the focal lemurs ingested *Vitis* cinerea leaves, which may contain large amounts of hydroxyprolin (App. II.2).

Furthermore, the saliva of Coquerel's sifakas was found to be rich in proline (Glander, pers. comm.), which may be interpreted as an important adaptation to a tannin rich diet through evolution. Principally, proline-rich proteins occur in saliva, but their actual quantity strongly depends on the animal species and the temporal nutrition. For instance, in human saliva, proline-rich proteins comprise about 70 % of the total proteins (Butler and Rogler, 1992), while their content is inducible in rodents (rats and mice), and completely absent from the saliva of hamsters (Baxter *et al.*, 1997), which causes increased mortality rate when hamsters are fed tannins (Butler and Rogler, 1992).

The interaction of tannins with enzymes, e.g. their enzyme inhibiting properties, are also still subject to controversial discussion (Horigome *et al.* 1988; Makkar *et al.*, 1988; Butler and Rogler, 1992; Hagerman 1992; Tebib *et al.*, 1994-95). In some animals, dietary tannins do inhibit enzymes including proteases, lipases and glycosidases, but increase enzyme activity or induce enzyme synthesis in others. It was also found that interactions of proteins with tannins were strongly influenced by pH and detergents (Hagerman, 1992; Tebib *et al.*, 1994-95). Alkalinity of the duodenum, the main site of nutrient absorption in monogastric mammals, prevents the polyphenolic compounds from binding to proteins. Additionally, the pancreatic biliary juice acted as a detergent and prevented or reversed the tannin-inhibited enzyme activity in the rat intestine (Tebib *et al.*, 1994-95). Therefore, the observation of increased faecal nitrogen, which was first attributed to the protein of dietary origin, is now thought to originate from endogenous proteins within the saliva, the gut mucosa or within secretions of the digestive tract (Butler and Rogler, 1992; Tebib *et al.*, 1994-95).

The complexation between polyphenols and polysaccharides (McManus *et al.*, 1985; Haslam *et al.*, 1989) is another important interaction, that depends upon their molecular sizes, conformational flexibility of the polyphenol ligand and the ability of the polysaccharide to form pores, holes or crevices either preformed in their three-dimensional structure or developed *in situ* during the complexation process. Unlike the complexation with proteins, complexation with polysaccharides is largely independent of the pH (4-7) (Haslam *et al.*, 1989). This seems important for interactions with both, the mucosal and the plant polysaccharides, i.e. plant mucilage. As tested in this study (chapter C.V), *R. copallina* leaf tannins did not disturb the gelling properties of *A. julibrissin* leaf polysaccharides. Hence, within the gut, synergistic antidiarrhoeal effects can be expected. Furthermore, a chemical interaction between tannins and the gastrointestinal mucus has been proposed by Verhaeren and Lemli (1986): the anti-secretory (i.e. the absorption of water) effect of gallotannins applied to the colon of guinea pigs even continued after application of rhein, which normally induces a secretagogue diarrhoea in the colon.

The ability of tannins to form complexes with multiple charged metal cations is another important interaction that also affects the functioning of enzymes. Chelating with metal ions of metallo-enzymes is one reason for their growth-inhibitory effects upon microorganisms, or may result in a depletion of essential metal ions (Scalbert, 1991).

The development of hemosiderosis in captive lemurs, that concerned one Coquerel's sifaka and several other lemur species at the San Diego Zoo (Spelman *et al.*, 1989; see necropsy reports), was attributed to a diet high in iron, high in ascorbic acid, and low in tannins. Correction of these parameters significantly improved their respective health conditions (Spelman *et al.*, 1989).

Both, the intestinal mucosal damage and bacterial translocation (in shocked animals) are reduced by the treatment with e.g. deferoxamine, an iron chelator and antidote against poisoning with iron (Berg, 1998), and with e.g. allopurinol, a xanthine oxidase inhibitor. Tannins possess heightened iron chelating properties. Including the other major plant compounds of this study, a reduction in the bioavailability of dietary iron is also reported for mucilaginous polysaccharides, as in the case of *Plantago* seeds (scientific information). Xanthine oxidase inhibition is reported for tannins (Candan, 2003), and for quercetin and further flavonoids, that decreased the oxidative injury (Cos *et al.*, 1998; Nijveldt *et al.*, 2001).

With respect to the development of diabetes (compare necropsy reports), for the group of tannins, antidiabetic properties with a reduction of postprandial blood glucose levels are reported (De Kiesgen and Maurel, 1994; Shim *et al.*, 2003). (Secondarily, diabetes in turn may cause diarrhoea (Ruppin, 1994; Ruppin and Soergel, 1994)). In addition, tannins have also been revealed to possess beneficial effects against other metabolic diseases such as hypercholesterolaemia, hypertriglyceridaemia and hypertension (De Kiesgen and Maurel, 1994). For gallic acid and galloyl gallic acid a triglyceride-decreasing potency was found (Iwata *et al.*, 1997).

Discussion on antimicrobial effects

The decision for the three bacterial test strains included in the preliminary assays, *Salmonella* E., *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, was based on their importance with several respects. *Salmonella* are pathogens that affect predominantly the gastrointestinal tract causing a devastating diarrhoea. *St. aureus* is able to multiply in food and to secrete a toxin. This food poisoning may result in a toxin diarrhoea with no bacterial adherence or penetration through gastrointestinal barriers (Thorne, 1986). Furthermore, *Staphylococcus* and *Pseudomonas* strains are both facultative pathogens and hence responsible for a range of opportunistic infections (e.g. hospitalism). They are involved in many inflammatory diseases, which in part have their starting point by penetrating through intestinal barriers. Vice versa, a damaged gut mucosa may serve as a port of entry.

Apart from humans, *St. aureus* occurs in nearly all species of animals. *Staphylococcus aureus* strains are able to cause dermatitis, tonsillitis, endocarditis and inflammations of the respiratory tract like pneumonia. *Pseudomonas aeruginosa* causes wound infections, endocarditis, inflammations of the urogenital and respiratory system, pneumonia and meningitis. Once penetrated through intestinal barriers, both, *Ps. aeruginosa* and *St. aureus* may affect multiple organs and thus lead to a generalised septicaemia or a lethal septic shock with multiple organ failure and lethal outcome (Pschyrembel, 1986; Bisping and Amtsberg, 1988; Tsai *et al.*, 1996). For these patients with multiple diseases, that are immunodeficient and / or hospitalised, or patients with a damaged intestinal mucosa e.g. after a history of diarrhoea, are rather predestined victims to opportunistic germs (Pschyrembel, 1986; Bisping and Amtsberg, 1988; Tsai *et al.*, 1996; Dingermann and Loew, 2003).

This pathogenic mechanism of bacterial infections seems interesting in view of symptoms described in the necropsy reports of captive sifakas. The finding that the most preferred leaf species *R. copallina* yielded superior antibacterial results may be interpreted as a preventive measure in regard to gastrointestinal mucosal damage and subsequent septicaemia as indicated in the necropsy reports of captive sifakas.

In this study, during the *in vitro* screening on the antimicrobial efficacy of the preferred leaf species by the sifakas (Tab. C.22), *R. copallina* leaves were effective against different *Staphylococcus aureus* strains, *Corynebacterium minutissimum*, *Bacteroides fragilis* and *Helicobacter pylori*. An increased antibacterial activity was found against *Pseudomonas aeruginosa* and *Corynebacterium acnes.* To better understand this finding, the bacterial species will be briefly characterised in the following.

The genus *Corynebacterium* contains numerous species of which only a few are pathogenic to animals or man (Bisping and Amtsberg, 1988). According to its name *C. acnes* occurs occasionally in acne pustules (Pschyrembel, 1986).

Members of the genus *Bacteroides*, which are obligate anaerobic gram-negative rods, represent the majority of the resident intestinal flora in humans and animals. They also form an important constituent of the oral and skin flora and they inhabit the mucosa of the genital tract (Bisping and Amtsberg, 1988; Onderdonk, 1998). There are enormous populations that predominate in the human tract and are highly involved in processes such as polymer hydrolysis and energy-yielding metabolism. For instance, gastric mucin is one endogenous source of carbon and energy for *Bacteroides fragilis* in the human intestinal tract (Savage, 1986). *Bacteroides* are relatively non-resistant outside the host’s body.

Bacteroides fragilis is another facultative pathogen which may exert a pathogenic action in body cavities and may involve various organ systems, especially multimorbid persons. Apart from various infections, a septicaemia may be generated (Pschyrembel, 1986). Together with other obligate anaerobes or facultatively anaerobic species, they may produce a purulent necrotising inflammation and the formation of abscesses. In such mixed infections, which are generally of endogenous nature, the synergistic interaction of various pathogenic types plays a potentiating role (Bisping and Amtsberg, 1988). With respect to the ecological equilibrium of the gastrointestinal microflora it should be noted that *R. copallina* leaves were effective against *Bacteroides fragilis*.

Helicobacter pylori is a spiral-shaped, anaerobic gram-negative bacterium, which colonises the epithelial surfaces of the gastric mucosa. In humans it is a major cause of gastrointestinal diseases such as acute and chronic gastritis, peptic ulcer disease and gastric cancer (Matsukura *et al.*, 1995; Ching and Lam, 1995; Taniguchi *et al.*, 1995; Jaspersen, 1996; Slomiany *et al.*, 1997). It is now accepted that *H. pylori* causes gastritis in more than half of the world’s population and that about 95 % of duodenal ulcers and 70-80 % of gastric ulcers result from infections with *H. pylori*. Furthermore, this organism may have a causal role in perhaps up to 60-70 % of gastric cancers (Niemelä *et al.*, 1995; Jaspersen, 1996; Lamouliatte *et al.*, 1996; Lee, 1996). Furthermore, *Helicobacter* spp. have been associated with enterocolitis and inflammatory bowel disease in humans and animals. A novel *Helicobacter* species was found in faecal samples of *Saguinus oedipus*, the cotton-top tamarins, that easily succumb in captivity to chronic colitis and subsequent colon cancer (Saunders *et al.*, 1999), yet the pathogenesis is still unclear.

The antibacterial activity of the *R. copallina* leaf extract against *H. pylori* found in this study (Tab. C.22) concurs with reports on *Rhus javanica* gall extracts and gallic acid, which both inhibited *H. pylori* growth (Bae *et al.*, 1998).

At present, it should be emphasised that the test system and the test conditions employed have influence on the obtained results, and *in vitro* conditions may not necessarily mirror *in vivo* conditions. Furthermore, leaf extracts containing a variety of secondary plant compounds often react differently than isolated single compounds. Additive, synergistic or antagonistic effects of a mixture of leaf compounds are difficult to assess, but have a major impact on the resulting effect e.g. on the digestive tract. Thus, a comparison of the results obtained in this study to antimicrobial properties reported in the current literature, demands a sophisticated evaluation. It should be emphasised that the results of the *in vitro* assays do not claim a complete inactivity for *A. julibrissin* leaves when ingested.

For example, in this study no activity could be found for *A. julibrissin* leaf extracts, albeit it has already been reported that quercetin inhibits *H. pylori* in a concentration dependent manner (Beil *et al.*, 1995). Quercetin was found in this study to be the major flavonoid aglycone in *A. julibrissin* leaves (Tab. C.16, p. 125). Beil *et al.* (1995) proved under experimental conditions that some flavonoids, including quercetin, inhibited the acid production in parietal cells by complexing with ATP which inhibited the H^+/K^+-ATPase, the gastric proton pump. As growth inhibition of *H. pylori* and blockage of acid production are crucial factors to the pathogenesis of gastric lesions, drugs containing flavonoids may have a therapeutic potential for the treatment of gastrointestinal diseases associated with *H. pylori* infection (Beil *et al.*, 1995).

In this study, the *R. copallina* leaf extract tested negative under experimental conditions against *Bacillus subtilis*. *Bac. subtilis* is another opportunistic bacterium that may cause various inflammatory diseases, e.g. meningitis and pneumonia, and may lead to sepsis. Gallic acid methyl ester has been reported to possess antibacterial properties against a range of gram-positive and gram-negative microbes causing inflammatory diseases of the gastrointestinal and respiratory tract (Kayser and Kolodziej, 1997; Kolodziej and Kayser, 1998). Gallic acid was reported to show growth-inhibitory effects against *Bac. subtilis* (Majinda *et al.*, 1997; Aaku *et al.*, 1998). Both compounds, gallic acid methyl ester and gallic acid, have been found in this study to occur in the *R. copallina* leaves. Therefore, it may be hypothesised, that *in vi*vo gallic acid contributes to the antibacterial potency of the gallotannins after successive hydrolysis within the gastrointestinal tract.

Further reports from the current literature corroborate the findings on antibacterial properties of the hydrolysable tannins and their monomers as indicated in this study, and give reason to expect synergistic effects between single leaf compounds when released during the gut passage, and the polymeric phenolics. For instance, the antibacterial effect of gallic acid against *St. aureus* found in this study (Tab. C.22), agrees with reports of Richards *et al.* (1994) and Majinda *et al.* (1997). According to Ahn *et al.* (1994; 1998), a methanolic extract from galls of *Rhus chinensis* as well as isolated methyl gallate and gallic acid showed selective and dose-dependent growth-inhibitory effects *in vitro* on bacteria of the gastrointestinal microflora. While harmful bacteria such as *Clostridium perfringens* (associated e.g. with sudden death and diverse gastrointestinal diseases in humans), *Cl. paraputrificum*, *Eubacterium limosum*, *Bacteroides fragilis*, *St. aureus* and *E. coli* were strongly and dose-dependently inhibited by methyl gallate and gallic acid, beneficial bacteria like bifidobacteria and lactobacilla remained largely unaffected.

Additionally, antibacterial effects against *E. coli*, *Klebsiella pneumoniae* and *Bacillus cereus*, and aflatoxin production inhibiting effects in *Aflatoxin* strains were reported for simple phenolics such as p-hydroxy benzoic acid, p-coumaric acid, protocatechuic acid and the flavonol aglycone quercetin (Aziz *et al.*, 1998), which have also been isolated from *A. julibrissin* and / or *R. copallina* leaves in this study (Tab. C.8, p. 107). Protocatechuic acid is reported to exhibit similar activities compared to gallic acid (Kroes *et al.*, 1992; Yun-Choi *et al.*, 1993), and thus, additional or synergistic effects can be expected between simple phenolics and polyphenolic compounds.

With respect to antifungal properties, no antifungal effects could be found for *R. copallina* leaf extracts in this study. However, in disc diffusion assays impregnated with methanolic extracts from *Rhus glabra* branches, it was revealed to possess moderate antifungal activities against several fungal strains at high concentrations (McCutcheon *et al.*, 1994). Furthermore, in this study *R. copallina* leaf extracts did not show any activity against the *E. coli* strains tested, although e.g. tannin albuminate has since long been successfully installed as antidiarrhoeal to prevent or alleviate traveller's disease which is predominantly caused by *E. coli* strains (Raedsch *et al.*, 1991). Once again this reveals the discrepancy between *in vitro* assays and their relevance for *in vivo* conditions.

Discussion on toxin inhibiting properties

The cholera toxin causes a massive diarrhoea[1]. Even after a brief intraluminal exposure to the toxin, directly or indirectly, a marked secretion of electrolytes, glycoproteins and fluids is induced into the small intestine (Goodgame *et al.*, 1973; Sherr and Mertens, 1979; Townsend *et al.*, 1981; Hör *et al.*, 1995). When untreated, cholera typically leads to death by dehydration (Schultz, 1984; Voet and Voet, 1990). *Vibrio cholerae* neither invades nor damages tissues, but merely colonises the intestine as does *E. coli*. However, *V. cholerae* infection or its toxin, do stimulate the humoral immune response, innate cells and inflammatory mediators within the host (Qadri *et al.*, 2004).

The electrophoretic cholera toxin binding assay in this study revealed that *R. copallina* tannins are capable of directly interacting with the toxin. As already evidenced for oligomeric proanthocyanidins (Hör *et al.*, 1995), the principle of binding to enterotoxins produced by pathogenic bacteria and thereby inactivating the toxins, can obviously be extended to *R. copallina* gallotannins which the sifakas are exposed to in this study. Similarly to the results presented by Hör *et al.* (1995), it was shown that *R. copallina* tannins bind to the enzymatically active A subunit of the cholera toxin which leads to a dose-dependent decrease in the A band as evidenced by gel-electrophoresis (Fig. C.13 and Fig. C.14). Obviously, seasonal influences affect the binding capacity of *R. copallina* tannins. The isolated compounds R16 from *R. copallina* leaves, p- and m- digallic acid methylester, are incapable of inactivating the cholera toxin. As the mode of action was not investigated further, the reason has yet to be elucidated. The molecular weight and stereochemical factors may be crucial. The reason, as to why commercial tannic acid is far less active in this assay than *R. copallina* tannins, is yet another interesting point which needs further investigation. As tannin albuminate consists of tannin that is already bound to proteins, this *in vitro* assay may be inappropriate in order to determine its activity as *in vivo* deliverance from the protein complex precedes any activity within the gastrointestinal tract.

However, the binding assay to cholera toxin revealed to be a useful means to elucidate possible antidiarrhoeal and beneficial gastrointestinal effects of *R. copallina* leaf tannins. This

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[1] The diarrhoea caused by the Cholera toxin is described as rice-water stool: "a clear solution with flecks of visible mucus, resembling water in which rice has been washed", (Sherr and Mertens, 1979).

in vitro assay may give another hint to reasons why *R. copallina* leaves have been selected so heavily by the Coquerel's sifakas as shown in this study.

Polysaccharides

The third group of compounds with expected beneficial effects for the gastrointestinal tract, are the polysaccharides (i.e. the pectin-like mucilage; compare chapter C.V) discovered in *A. julibrissin* leaves during this study. Generally, mucilaginous and gelling drugs are utilised for their soothing and adsorbing properties (Steinegger and Hänsel, 1988).

Externally, gelling agents administered to the skin exhibit cooling effects. Sterile solutions of pectins are utilised externally for wounds and skin lesions because of their soothing, haemostyptic and indirect antibacterial effects. Acidic polysaccharides in general exhibit hyaluronidase-inhibiting and thereby cause (indirectly) antibacterial effects, while the bacterial propagation is inhibited within the human and animal tissue (Steinegger and Hänsel, 1988). Eyedrops containing *Tamarindus indica*[1] seed polysaccharides are utilised against the "office eye syndrome" and other forms of the dry eye. These polysaccharides are integrated into the mucin layer of the human eye and are thus able to stabilise and regenerate the lacrimal film (Bruhn, 2005).

Internally, mucilages and pectins are utilised for their soothing, embedding and adsorbing properties. Depending on the mucilage structure and physico-chemical properties, mucilages are utilised for coughs (in lozenges and teas), respiratory disorders such as bronchial catarrh, and for gastrointestinal disorders such as diarrhoeal diseases, gastroenteritis, ulcers and hyperacidity of the stomach (Steinegger and Hänsel, 1988).

The activity of plant polysaccharides on the gut motility depends on their structure type, species-specifically on the gut (section) and on the presence of water. The binding of water, (cationic and organic compounds) is thought to increase stool consistency, while secretions are absorbed and pathological degradation products are neutralised in bouts of diarrhoea. On the contrary, an increase in the filling pressure is beneficial in bouts of obstipation. Both, the swelling and fluid and electrolyte absorbing properties, and conversely increasing the filling pressure, can exert normalising effects on the gut motility and transit time. Furthermore, depending on the contents of water, these compounds either constipate with little water or lubricate with a great deal of water (Steinegger and Hänsel, 1988; Dingermann and Loew, 2003). However, constipating and laxative agents act differently depending on the animal species[2] and their respective gastrointestinal tract.

Moreover, indigestible polysaccharides, including their degradation products, the short-chain fatty acids, exert curative effects on epithelial cells, influence the microbial ecosystem within the gut by e.g. lowering the intraluminal pH, and are used as fuel for nutrient and energy supply. Especially pectins and gums are rapidly and fully fermented, yielding different amounts of short-chain fatty acids (Steinegger and Hänsel, 1988; Dingermann and Loew, 2003; Campbell *et al.*, 2004b; for details see chapter C.I). Acid hydrolysis of mucilage of *Plantago* seeds (arabinoxylanes, classified as hemicellulose) in the stomach yields monomer, terminal arabinose residues, while products of lower molecular weight including short-chain fatty acids generated through enzymatic degradation by e.g. *Bacteroides* strains, enhance the colonic bacterial growth (Hensel and Hose, 2001; Rimpler, 1999). Hence, the benefits of indigestible polysaccharides such as mucilages and pectins (otherwise classified as dietary fibres, see chapter C.I) are of dual character, one nutritional and one pharmacological on epithelial cells and the microbial flora and ultimately onto the gastrointestinal integrity.

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[1] Leaves and fruits of *T. indica* are preferred food items by wild *P. v. verreauxi* in the south of Madagascar (Ny Andry Ranarivelo, pers. comm.).

[2] In this respect, herbivores react differently from carnivores and omnivores such as mice (Schmid, 1952).

Drugs such as Plantaginis ovatae semen, and Plantaginis ovatae testa[1] (e.g. Metamucil powder, Mukofalk® granulate), are utilised as basal and adjuvant therapy for acute and chronic obstipation, diarrhoea, irritable bowel syndrome with alternating obstipation and diarrhoea, diverticulitis, haemorrhoids, Crohn's disease and in support in metabolic diseases such as diabetes, lipaemia and adiposis, as well as in pregnancy (scientific information of Dr. Falk Pharma GmBH; Tomoda *et al.*, 1990; Dingermann and Loew, 2003).

While the neutral mucilage of *Plantago* species is predominantly indicated for the irritable bowel syndrome and as an adjuvant in diarrhoeal diseases, pectins (e.g. apple pectin) are predominantly utilised for acute and chronic diarrhoeas (Steinegger and Hänsel, 1988; Dingermann and Loew, 2003; Rote Liste, 2005). Additionally, for pectin-like and acidic polysaccharides a strong inhibition in gastric lesion formation and preventive anti-ulcer activities have also been found, showing a dose-dependent activity (Yamada *et al.*, 1991; Sun *et al.*, 1992). As with most other indigestible polysaccharides which need gut microflora for degradation, pectins lower both the blood serum cholesterol levels and postprandial blood glucose levels (Steinegger and Hensel, 1988).

In this study, the plant mucilage of *A. julibrissin* leaves was characterised as pectin-like rhamnogalacturonan (see chapter C.V). Therefore, protective effects onto the mucus layer are to be expected. *In vitro*, pectin showed best creeping properties in that it covered the epithelial surface of the small intestine and colon, although it did not protrude between the microvilli under these conditions (Janssen, 1982; Janssen and Queisser, 1982). However, pectin appeared to interact with the bacterial colonisation (Larsen, 1981). Furthermore, a dependence of the gel viscosity on pH and temperature was found for pectin (Janssen and Queisser, 1982).

In regard to the interaction between pectins and tannins, there are experiments (Gebhardt, 1935), that plead for opposite effects of pectin and tannin albuminate in healthy and diarrhoeal experimental animals with respect to transit time and stool consistency: tannin albuminate enlarged the gastrointestinal transit time and increased stool consistency by reduced water content, while pectin accelerated gut passage time and diminished stool consistency by increasing water content. Furthermore, a mixture of both, pectin and tannin albuminate, showed properties which resembled more the tannin than the pectin moiety. As the sifakas selected a diet mixed in both groups of chemical compounds, normalising effects on their gut motility seemed evident.

It should be noted, that additional effects were described for polysaccharides depending on their chemical structure (e.g. anionic charge, steric factors), including those on the immune system (Yamada *et al.*, 1986; 1987; Kiyohara *et al.*, 1988; Tomoda *et al.*, 1990; Classen, 2005). For instance, polyanion structures revealed *in vitro* an increased activity compared to neutral polysaccharides. Furthermore, for various polysaccharides a more or less pronounced antitumor activity has been found with branched glucans and arabinogalactanes being superior to acidic and pectic polysaccharides (Kraus *et al.*, 1986; Müller *et al.*, 1989; Yamada *et al.*, 1990). In this context it is notable that *in vivo* experiments with a fraction of crude polysaccharides from *A. julibrissin* (neither type of polysaccharide, nor plant organ was specified by the authors) revealed a relatively strong antitumor activity (Moon *et al.*, 1985), albeit these effects seem less relevant for the sifakas.

However, a conclusive evaluation of antidiarrhoeal and beneficial properties of the *A. julibrissin* leaf polysaccharides onto the gastrointestinal tract of the sifakas needs further investigation.

[1] Psyllii semen (DAB 9) from *Plantago psyllium* (syn. *P. afra*) and *P. indica* (syn. *P. arenaria*); and Plantaginis ovatae semen (DAB 9) and Plantaginis ovatae testa (DAC 1986; Ispaghula husks) from *P. ovata* (syn. *P. ispaghula*).

3.5 Relevance of food plant selection and daily intake of the major plant compounds

The contents of the tannins and flavonoids as determined for each leaf species were referenced with the daily intake of the sifakas, respectively (C.VI.2.3). The quantities of the major plant compounds supplied by the different leaf species were summed each day and averaged for each animal group over the number of observation days. Taking into account the body weight of each individual lemur, the intake of tannins and flavonoids was calculated per kg bodyweight, or additionally for each individual sifaka (Tab. C.24; Tab. C.25). In these calculations, all leaf species supplied by the browse and those selected in the forest were included. This allowed for the amounts of ingested leaf species to then be converted into the amounts of ingested plant compounds. This is the first study design that includes the feeding data of focal animals collected under "field-like" conditions.

Prior to any dosage calculations, it should be noted that in the group D&V during the spring season, Valentinian was a subadult (pre-puberty) and Drusilla was a juvenile at time of data collection. In the autumn, Gordian was a juvenile. For calculations related to an individual's bodyweight, it should be noted, that sifakas weigh far less than humans and in part it seems preferable to refer dosages per animal, irrespective of their weight. The average daily intake of gallotannins and flavonoids ingested by the sifakas is now evaluated in comparison to therapeutic dosages common within human medicine (Tab. C.24; Tab. C.25).

Tannins

Dosage calculations for tannins (Tab. C.24) are based on Tannalbin® tablets (Dr. Rentschler Arzneimittel GmBH) containing tannin albuminate, a gallotannin-protein complex in the ratio 1:1, which has since long been established in combating diarrhoea in human medicine. According to the scientific literature, in case of acute diarrhoea, every 1-2 hours 1-2 tablets (à 500 mg tannin albuminate) or 2-4 capsules (à 250 mg tannin albuminate) are recommended for adults, which correspond to a minimum of 250 mg and a maximum of 4-6 g pure gallotannins per day. As daily travel prophylaxis 500 mg gallotannins are recommended for adults.

Maximum daily dosages for children and babies depend upon the age with 3 g gallotannins for children aged 6-14 years, 1.5 g tannins for children aged 1-6 years and 750 mg for babies older than three months, respectively.

Tab. C.24: Averaged daily intake of gallotannins [g] per individual sifaka, and recommended dosages (related to pure gallotannins) for human adults.

	spring				autumn			human
	M	T	D	V	J	D	G	adults
intake [g]	4.94	3.78	4.65	6.20	1.61	1.77	2.48	0.25 - 6

If these recommendations are compared to the sifakas[1] (Tab. C.24), all animals consumed gallotannins in relevant therapeutic dosages, although a clear seasonal difference becomes visible. While in spring all study animals consumed high averaged daily dosages irrespective of their age, between 3.78 g gallotannins (Tiberius) and 6.20 g (Valentinian, subadult), far less tannins were ingested in the autumn. The adults Julian and Drusilla ate on average 1.61 g and 1.77 g gallotannins, respectively, and Gordian consumed approximately 2.48 g. This trend seems more to reflect seasonal constraints and limits in the food supply (offered by the staff) than voluntary food choice. Furthermore, the gallotannin content was reduced in the autumn

[1] With respect to dosage calculations, juvenile sifakas correspond to human infants (1-5 years); subadult sifakas correspond to older children (6-12 years); and adult sifakas correspond to children > 12 years and human adults.

leaves (compare Tab. C.13, p. 119). Conversely, in neither season were *R. copallina* leaves provided by the staff *ad libitum*. Gordian ate all *R. copallina* leaves he could access and reached the recommended maximum human dosages, yet he appeared to desire more. In this context it is notable, that gallotannins possess an enormous therapeutic broadness, indicating a largely non toxic drug (Scholz, 1994; Dingermann and Loew, 2003).

Flavonoids

The group of flavonoids is abundant in many food and medicinal plants within human nutrition and medicine. Their daily intake with diet varies greatly and is presumed to range from approximately 50 mg to 1 g (Middleton and Kandaswami, 1994; Wagner, 1999).

Tab. C.25: Average of flavonoid glycosides and major aglyca daily ingested by each respective group [g] and per bodyweight [g / kg].

major compounds	ingested amounts M&T g	M&T g / kg	D&V g	D&V g / kg	J&D g	J&D g / kg	G g	G g / kg
quercitrin	0.50	0.06	0.58	0.10	0.19	0.02	0.05	0.02
total flavonoids	1.90	0.22	2.18	0.37	1.04	0.12	0.27	0.09
myricetin	0.03	0.00	0.07	0.01	0.05	0.01	0.01	0.00
quercetin	0.59	0.07	0.61	0.10	0.17	0.02	0.06	0.02
kaempferol	0.07	0.01	0.08	0.01	0.03	0.00	0.01	0.00
total aglyca	0.79	0.09	0.87	0.15	0.35	0.04	0.13	0.04

Since no antidiarrhoeal in human medicine explicitly consists of flavonoids (Rote Liste, 2005), no reliable dosage recommendations are available for this specific indication. So, for dosage comparisons of the flavonoid intake within the sifakas, the data from antidiarrhoeal experiments with quercitrin, quercetin and additional flavonoid glycosides and aglyca were used (Gálvez *et al.*, 1993a, b; Gálvez *et al.*, 1995; Rao *et al.*, 1997). Dosage recommendations for flavonoids with indications other than diarrhoea were also noted (Rote Liste, 2005). But dosages of remedies containing flavonoids are recommended for adults and adolescents older than 12 years of age irrespective of their actual bodyweight (Rote Liste, 2005), which make comparisons difficult, especially of juvenile sifakas to children of comparable age. However, the focus was on quercitrin and additional quercetin glycosides, since quercitrin was the predominant flavonol glycoside and quercetin the most abundant aglycone analysed in the food plants of the focal lemurs.

According to the literature, orally administered quercitrin and quercetin were effective on gut motility, fluid and electrolyte secretion at dosages of 25 and 50 mg per kg bodyweight (Gálvez *et al.*, 1993a, b; Gálvez *et al.*, 1995), while intraperitoneally administered flavonoids to rats and mice were in part, active at lower levels. For instance, the small intestinal transit was delayed by apigenin at 25 mg per kg bodyweight, while kaempferol, myricetin and rutin were active at 12.5 mg per kg bodyweight (Di Carlo *et al.*, 1993). Intraperitoneally administered rutin showed gastroprotective effects in rats at a dosage of 50 mg per kg bodyweight (Rao *et al.*, 1997).

Compared to the quercitrin dosages of 50 mg per kg bodyweight which were orally administered (Gálvez *et al.*, 1995), the self dosages of the sifakas in the spring came close to or exceeded this value, while the animals in the autumn ranged below this dosage (see Tab. C.25). However, the amount of “total flavonoids” ingested exceeded this value by all animals.

Considering remedies with flavonoids (with medicinal indications other than diarrhoea), there are different recommendations for orally administered dosages of flavonoid glycosides (Rote Liste, 2005). For instance, quercitrin constitutes the major flavonoid glycoside in *Solidago* *virgaurea* extracts, for which diuretic, antiphlogistic, analgesic, antifungal and mild antispasmodic effects are reported (Bongartz, 1995; Van Wyk and Wink, 2004). Most pharmaceutical preparations containing goldenrod extracts are used for their diuretic and antiphlogistic properties to treat nephritis, nephropathies (in pregnancy), renal oedema, renal hypertension (e.g. Solidagoren® N, liquid), as well as in higher dosages for urinary and kidney gravel and stones (e.g. Cysto Fink Mono, capsules; Urol® mono capsules; Rote Liste, 2005). Calculated for quercitrin, the daily recommended dosage for adults varies from approximately 4 mg (Solidagoren® N, with 1 mg / ml fluid extract from *Solidago virgaurea*; Dr. Klein (Pharma), pers. comm.) to over 100 mg (Urol® mono) depending on the remedy (Bongartz, 1995; Rote Liste, 2005).

Rutin, another quercetin glycoside (rutoside, quercetin-3-rutinoside), and its commercially used herbal source *Fagopyrum esculentum*, are extensively studied for their pharmacological effects and dosage recommendations. Rutin and its derivatives are used as mono-preparations for the treatment of vascular disorders such as heavy legs, increased permeability of vessels, varicosis, thrombophilia and haemorrhoids (Rote Liste, 2005). This vascular protection (achieved by reduced permeability and compression), vasoconstrictor and anti-haemorrhagic effects improved the vascular resistance in pregnancy, in hypertonic patients, and retinal haemorrhages in diabetes (Dingermann and Loew, 2003). Recommended dosages depend upon the degree of ailment and range from e.g. 50 mg rutoside (Rutin-capsules), over 300 mg rutoside (Rutinion® 50 mg, tablets), 600 mg rutoside (Rutinion® FT 100 mg, tablets), 500-1000 mg O-(ß-hydroxy-ethyl)-rutoside (Venoruton® Active and Venoruton® Intens, tablets), and 300-1800 mg troxerutin (Veno SL® 300, capsules) up to 3 g in radiological therapy (hydroxy-ethyl-rutoside; Venoruton® Active / Intens, tablets) (Rote Liste, 2005).

Compared to the individual focal lemurs, in the spring season, the animals averaged daily ingested levels between 220 mg (Tiberius) and 330 mg (Valentinian) of quercitrin irrespective of their age. On the contrary, in the autumn season, the adult animals consumed on average daily 100 mg (Drusilla) and 90 mg (Julian), respectively and the juvenile Gordian consumed an average of 50 mg. As previously stated for the gallotannins, the difference between the spring and autumn dosage is likely to reflect seasonal constraints in flavonoid contents within the food supply provided by the staff than in more voluntary food choice. Although the contents of quercitrin increased in *A. julibrissin* leaves from spring to autumn, the overall contents of flavonol glycosides decreased (Tab. C.13, p. 119). This overall decrease in flavonoid glycosides was also found for the other food plants analysed. When provided, autumnal *A. julibrissin* leaves were less frequently consumed compared to those same leaves in the spring, presumably due to the predominance of old leaves. However, in both seasons (spring and autumn) quercitrin was consumed in therapeutic dosages as recommended for humans, and no clear dependence on the sifakas' age could be determined.

Since quercetin as an aglycone moiety seems to be important for the pharmacological effects of quercetin glycosides for the animal host (Fanning *et al.*, 1983; Gálvez *et al.*, 1993a), dosage recommendations for quercitrin and rutin as glycosides were converted into quercetin aglycone equivalents [1]. Therefore, the total amount of quercetin aglyca could be taken into account, and that also included the other quercetin glycosides other than quercitrin which were contained in the food plants and were ingested by the sifakas.

In human medicine, daily recommended dosages of orally administered quercetin range from 3 mg (contained in Solidagoren® N), over 145 mg (contained in Rutinion®, tablets) to even 1.4 g (corresponding to 3 g O-(ß-hydroxy-ethyl)-rutoside in Venoruton® Active / Intens). For a hypothetical 60 kg person, a minimum of approximately 0.06 mg with a maximum range of 24 mg quercetin per kg bodyweight lies within the therapeutic scope of pharmaceutical recommendations. But dosage recommendations are likely to be linked to the age of a person,

[1] converted by means of the molecular weights of the respective glycoside and its aglycone

not to the bodyweight, and in the case of a hypothetical 100 kg person, much lower values per bodyweight would result: just 0.02 mg up to 14 mg quercetin aglycone. However, this clearly shows the huge therapeutic broadness of flavonoids and their effects even in low dosages. For the extract from Fagopyri herba (from *Fagopyrum esculentum*, with rutin as leading flavonoid), the first toxic effects are reported to commence at a dosage of 14.7 g / kg bodyweight, the LD_{50} at 24.5 g / kg bodyweight, and the lowest lethal dosage at 215 g / kg in mice orally administered, respectively (Friederich *et al.*, 1999; Dingermann and Loew, 2003). Toxic effects found *in vivo* in different animal models e.g. for tannic acid and gallic acid in rabbits (Dollahite *et al.*, 1962; Pigeon et al., 1962), and for quercetin in mice and rats, seem to be of little importance for therapeutic dosages in humans (Steinegger and Hänsel, 1988; Dingermann and Loew, 2003), and for the sifakas of this study.

Quercetin intakes of the focal sifakas resemble those of quercitrin intakes (Tab. C.25). During the spring season, animals ingested a daily average between 260 and 350 mg of quercetin, irrespective of their age, while in the autumn, roughly 85 mg was eaten by the adults and just 60 mg by Gordian.

Considering the intake of total flavonoids referenced to the individual focal sifakas, during the spring the animals ingested daily average levels between 830 mg (Tiberius) and 1240 mg (Valentinian). In the autumn, Drusilla and Julian (with outdoor access) ingested 550 mg (Drusilla) and 500 mg (Julian) respectively, whereas Gordian amounted to a mere 270 mg.

It can be concluded that the quantity of total flavonoids ingested as well as the quantity of quercitrin and quercetin lay within the levels of therapeutic recommendations for humans. Within human medicine, dosage-finding assays revealed that the long-term treatment with even low dosages of e.g. rutin (40-60 mg rutin daily) yielded therapeutic success (Dingermann and Loew, 2003). Low dosed flavonoids (quercetin and quercetin glycosides) and other phenolic compounds (gallic acid and related compounds) showed prolonged pharmacological effects even after a single oral dose (Dorsch *et al.*, 1992; Neszmélyi *et al.*, 1993).

The daily dosages of flavonoids and tannins "dosed" by the sifakas can be classified as long-term therapy. No correlation to the animal's age could be ascertained.

Polysaccharides

The polysaccharides of high molecular weight were found only in *A. julibrissin* leaves (see chapter C.V), and these leaves were only fed as browse within the cages. As experiments on a direct quantification of *A. julibrissin* leaf mucilage failed, the determination of the swelling number (chapter C.V) was used as a reference method to compare the amounts of fresh *A. julibrissin* leaves eaten by the sifakas to therapeutic dosages of gelling agents as recommended within human medicine. Table C.26 presents the averaged daily intake of fresh leaves per individual (values were converted from Tab. B.9, p. 43) and the corresponding volume when balanced with the swelling number and referred to as dry leaf weight.

Tab. C.26: The averaged daily intake of fresh *A. julibrissin* leaves [g] per individual and the corresponding volume [ml] when balanced with the swelling number (QZ = 9 referred to dry leaf weight) are presented; recommendations for human adults are included [ml].

	spring				autumn			human
	M	**T**	**D**	**V**	**J**	**D**	**G**	**adults**
intake [g]	57	44	42	56	9.3	10	11	
volume [ml]	128	99	95	126	21	23	25	10 - 100

For dosage comparisons, some pectin containing remedies were considered.

Aplona® granulate contains apple powder[1], of which 20-35 g (4-7 sachets) are recommended for infants (1-5 years), and 25-40 g (5-8 sachets) for older children and adults on a daily basis for acute and chronic diarrhoeas. This corresponds to pectin quantities of 700 mg to 1.2 g for infants and 875 mg to 1.4 g for older children and adults. The swelling number for apple pectin was determined as 13-25 ml/g (Athenstaedt GmbH & Co. KG, pers. comm.).

Diarrhoesan® (liquid) contains a fluidextract of Matricariae flos and pectin. Recommendations for an antidiarrhoeal therapy yield a maximum of about 3-4 g pectin per day for older children and adults, about half the dosage for infants and about 1 g for babies (Dr. Loges & Co. GmbH, pers. comm.).

For *A. julibrissin* leaves eaten in May and the younger leaves eaten in October, a swelling number of 9 was determined, respectively (chapter C.V). Since there is no feeding data for July, the higher swelling numbers determined for leaves from July were not used in these calculations.

In the spring, individual focal animals ate approximately 50 g fresh *A. julibrissin* leaves irrespective of their age, whereas approximately 10 g were consumed in the autumn by each focal lemur. If the corresponding dry weights were balanced with the swelling number of the leaves (QZ = 9 ml/g), the animals in the spring reached an average of 112 ml and in the autumn 23 ml, respectively. Compared to the pectin containing remedies and the balancing of dosages prescribed with the swelling number of apple pectin (QZ = 13-25 ml/g), 10-100 ml are recommended for adults and older children, and 10-50 ml for infants, respectively.

It may be concluded, that all focal lemurs lay within (or exceeded slightly) dosage recommendations for adults irrespective of their ages. The clear seasonal difference in consumption of the polysaccharides obviously originated from the seasonal abundance of younger *A. julibrissin* leaves.

It may be further concluded, that pectin containing remedies are in good agreement with the *A. julibrissin* leaf mucilage, and this in three respects: physico-chemical properties (chapter C.V), indication, and dosages taken per individual.

Selectivity of the food

To evaluate the importance of *R. copallina* and *A. julibrissin* leaves as major source of tannins and flavonoids in the diet of the sifakas (Fig. C.15), the leaf composition eaten (weight %) was referred to the amount (weight %) of hydrolysable tannins and flavonoids supplied by these leaf species.

According to Fig. C.15, equal amounts of *R. copallina* and *A. julibrissin* leaves were ingested by Marcella and Tiberius during the spring, while slightly more *R. copallina* and less *A. julibrissin* leaves were eaten by Drusilla and Valentinian. Therefore, in both groups *R. copallina* accounted for the majority of hydrolysable tannins, 80 and 87%, respectively. Regarding the group of flavonoids, *A. julibrissin* leaves accounted for 64 and 55 % of total flavonoids ingested, respectively. Although in October far less *R. copallina* was eaten by Julian and Drusilla, (for absolute amounts of fresh leaves eaten see Tab. B.9, p. 43), *R. copallina* leaves still supplied 79 % of the hydrolysable tannins in the food. Due to the low percentage of *A. julibrissin* leaves, only 14 % of flavonoids originated from *A. julibrissin* leaves and 78 % were delivered by other leaf species. Gordian covered with a portion of 73 % *R. copallina* leaves his entire requirement for hydrolysable tannins. 13 % *A. julibrissin* leaves still supplied 30 % flavonoids.

This portion of mucilage is not depicted in Fig. C.15, as all mucilage ingested in captivity originated from *A. julibrissin* leaves.

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[1] 175 mg pectin are contained per sachet Aplona® granulate with 5 g apple powder each.

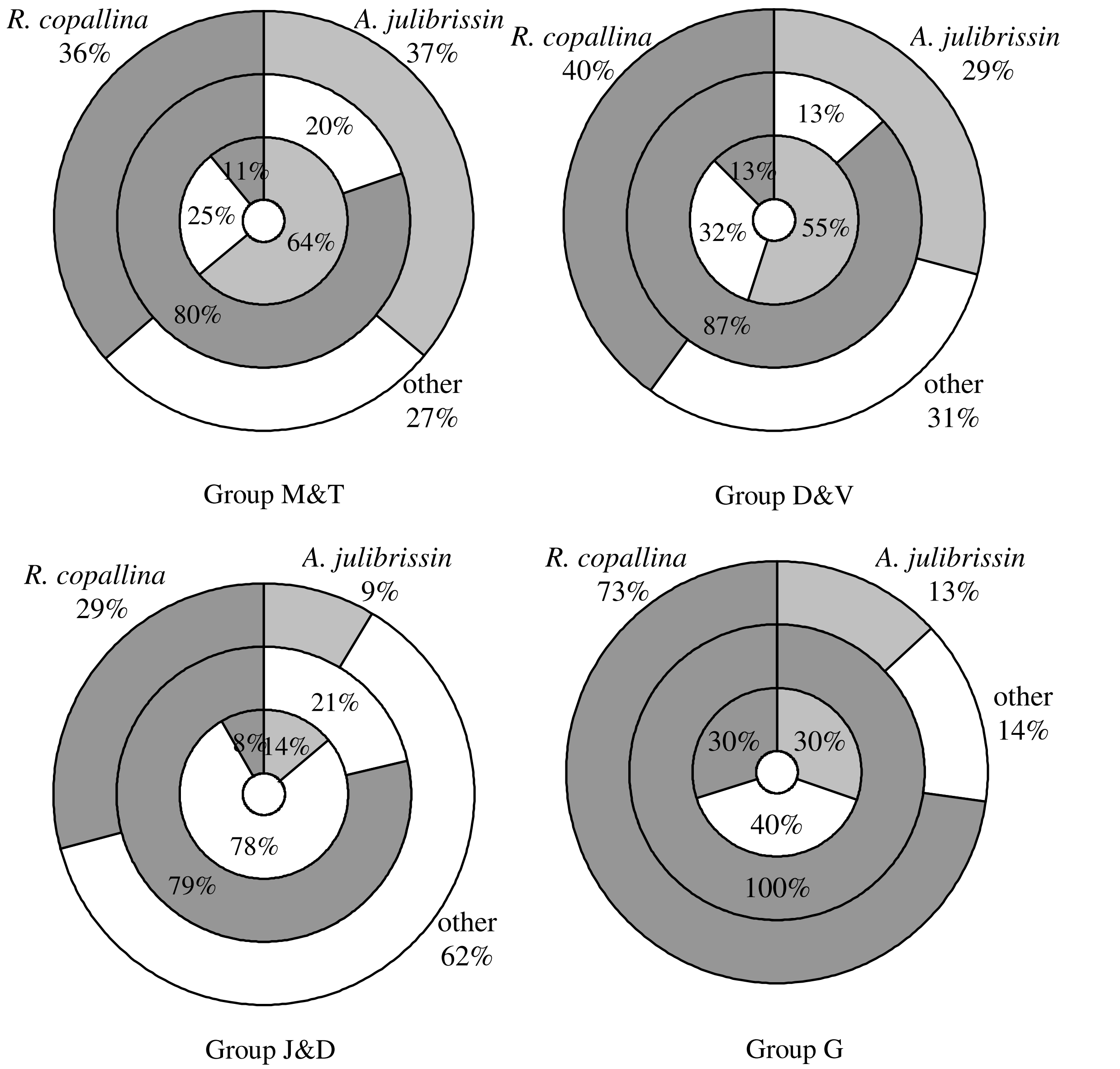

Fig. C.15: Food selection and supply of hydrolysable tannins and flavonoids by the different leaf species.
The food composition and the supply of the secondary plant compounds is given in weight %. Outer ring: leaf species eaten; middle ring: tannins ingested; inner ring: flavonoids ingested. Like colours correlate food plant sources with the supply of the respective compounds.

With respect to seasonal differences in contents of the three groups of compounds in the food plants combined with the food selecting behaviour of the sifakas, the greatest importance can be assigned to the group of hydrolysable tannins. They were heavily ingested throughout the year by selecting *R. copallina* leaves. Depending on the availability of *A. julibrissin* leaves, their inherent flavonoids and plant mucilage levels are expected to exert additional or synergistic beneficial effects resulting in an antidiarrhoeal triple therapy.

4 Discussion and conclusion

Secondary plant compounds exert a range of pharmacological effects and their utilisation for medicinal purposes is the origin of phytotherapy. However, the evaluation of the role of secondary plant compounds in the diet of the focal Coquerel's sifakas is a multifarious problem, since the daily ingestion of secondary plant compounds equals a long-term therapy. It appears that the lack of chemical compounds essential for their health has emerged as a health concern, especially the phenomenon of diarrhoea in captive Coquerel's sifakas and related indriid species. The preventive aspect of this treatment is mirrored by the finding that diarrhoea is not observed in wild sifakas (Garell, 1990). However, captive sifakas often fell ill when deprived of appropriate leaf species browse, without eating any detrimental or noxious foods. The curative aspect of this therapy is mirrored by the fact that animals recovered when having the opportunity to self-select special leaf species. Unfortunately sifakas often died of multiple infections and metabolic diseases when continuously deprived (see necropsy reports) of leafy browse. In the latter case no veterinary medicinal treatment was effective in stemming the final course of diseases after a history of recurrent and chronic diarrhoea.

Based on the selective feeding behaviour shown by the focal lemurs, a strong predilection for two food plants became clear, *R. copallina* and *A. julibrissin*. *R. copallina* leaves was the most preferred leaf species throughout the year and yielded most of the hydrolysable tannins ingested. *A. julibrissin* leaves yielded most of the flavonoid glycosides (characterised by a predominance of quercetin aglyca) and all polysaccharides ingested, (a pectin-like mucilage of high molecular weight). The amount of tannins and flavonoids ingested were supplemented by additional selected leaf species, which corroborate the selectivity in food plant choice.

Hydrolysable tannins possess a direct antidiarrhoeal effect as they are able to interact with the gastrointestinal mucous membrane, in order to reduce hypersecretory processes, to decrease the gut passage rate and increase stool consistency. With respect to the *in vitro* assays, extracts from *R. copallina* leaves, my findings showed antibacterial effects especially against opportunistic, facultative pathogenic bacteria, such as various *Staphylococcus* strains and *Pseudomonas aeruginosa*. Furthermore, other bacteria (*Corynebacterium minutissimum*, *C. acnes*, *Bacteroides fragilis* and *Helicobacter pylori*) were also affected by *R. copallina* leaf extracts. Moreover, tannin extracts from *R. copallina* leaves were able to bind to cholera toxin, which is still also a key cause for diarrhoeal diseases in developing countries. Since these effects can be attributed to the group of hydrolysable tannins, *R. copallina* leaves were the most effective leaf species in these assays.

As tannins are acknowledged to have an important impact on bacterial growth and colonisation, the resulting pharmacological effects of *R. copallina* leaf tannins are presumed to reduce the risk of an increased gastrointestinal permeability, bacterial translocation and maintain the equilibrium within the gastrointestinal microflora constant. In particular, opportunistic bacteria are able to cause severe inflammatory diseases with lethal consequences, if they have penetrated the barriers of a normally functioning immune system (Tsai *et al.*, 1996). Overall, the gallotannins ingested by the sifakas are thought to be able to develop their antidiarrhoeal potency throughout the entire gut like tannin albuminate (e.g. Tannalbin®). Simple phenolics found in *R. copallina* and *A. julibrissin* leaves (Tab. C.8, p.107) and those presumed to be released to some extent during gut passage from phenolic polymers (e.g. gallic acid) are expected to contribute to the antibacterial and antiseptic effects.

Flavonoids, especially of the quercetin aglycone type, are reported to exhibit spasmolytic and protective effects within the gut. They are found to reduce capillary permeability, to regenerate absorptive processes, to normalise the gut transit time and to restore the mucous membrane. These effects are attributed predominantly to *A. julibrissin* leaves due to their high contents in flavonoids, albeit these effects were not corroborated by assays in this study.

The polysaccharide, a pectin-like rhamnogalacturonan, was found to occur in *A. julibrissin* leaves and is expected to influence the gut motility, absorptive processes and to exert curative effects on epithelial cells and the gastrointestinal microflora. Mucilaginous compounds may influence bacterial growth, e.g. by influencing the intraluminal pH.

Overall, abnormal absorptive processes of the gut should be normalised and the mucosal barrier restored by both, the flavonoids and tannins. Gut motility itself seems to be influenced by all three groups of compounds. Interactions of the plant compounds with surface mucopolysaccharides of the gastrointestinal tract are expected for the tannins and the mucilaginous polysaccharides.

The conversion of the contents of these major leaf compounds within the food plant species into averaged daily dosages “self-dosed” by the sifakas, yielded relevant therapeutic dosages throughout the year, as utilised in human medicine. The different dosages ingested by the animals studied in spring and in the autumn appear to reflect seasonal fluctuations in leaf contents, and constraints within the food supply, rather than actual physiological needs. The huge therapeutic broadness of these three groups seems to allow for the large dosage variations as observed between the spring and autumn feeding behaviour.

The benefit from the three major groups of secondary compounds for diarrhoeal diseases is presumed to lie in their unspecified mode of action onto different processes within the gastrointestinal tract and associated organs.

With respect to human medicine, hydrolysable tannins are already well established for the prevention as well as for the treatment of acute and chronic diarrhoeal diseases. Additionally, mucilaginous compounds and pectins are acknowledged to normalise stool consistency. In experimental animal models, flavonoids create preventive and beneficial effects on the gastrointestinal tract and are helpful in diarrhoeal diseases, yet have not been established with this effect in human medicine.

Furthermore, it should be noted that a species-specific balance between the primary food constituents (proteins, fat, carbohydrates and dietary fibres) seems to be a precondition for the health of each animal species and humans (the dietary regime was changed prior to the onset of this study, see chapter C.I). In human medicine, the composition of primary food constituents is increasingly discussed in favouring or preventing the generation of metabolic diseases (such as diabetes, hyperlipidaemia and hypertension). With respect to the sifakas, a species-specific balanced diet in primary and secondary food components is essential for their health condition.

Based on the sifakas’ choice of secondary plant compounds inherent to their selected food plants, and the dosages taken, it may be concluded that the sifakas designed an antidiarrhoeal long-term triple therapy. This concept may also be beneficial for diarrhoeal diseases in humans.

Finally, it should be added that all three groups of major leaf compounds, hydrolysable tannins, flavonoids (especially of the quercetin aglycone type), and the pectin-like mucilage are rather ubiquitous in plant kingdom. These compounds are presumed to be in abundance within lemur ranges on the island of Madagascar and their absence within respective diets became a problem whenever sifakas left their natural habitats. Obviously, taste and smell are the senses by which sifakas select the “appropriate” plant species despite their foreign living conditions in zoos and parks outside Madagascar. The focal lemurs exhibited high selectivity in leaf choice, which translated to an active decision for high-yield leaves in tannins by the Coquerel’s sifakas, is a feature that appears to be common and well distributed among lemurs (Spelman *et al.*, 1989). Undoubtedly this heightened selectivity pleads for the hypothesis, that this trait provides a strong evolutionary adaptation whether they are residing in a foreign or a native environment.

D General conclusions

A common problem of Coquerel's sifakas is the development of diarrhoeal diseases in captivity. Their selective feeding behaviour with respect to leaf choice, the finding of three major groups of plant compounds (hydrolysable tannins, flavonoids and a pectin-like plant mucilage) within the selected leaves with their innate antidiarrhoeal effects as well as the therapeutic dosages ingested are compelling evidence that the Coquerel's sifakas consumed these compounds for their pharmacological effects and not for nutritional reasons. It is therefore plausible to conclude that phytotherapy is predestined for self medication especially by those animal species which are on the other hand "condemned" to cope with an assortment of pharmacological active compounds within their environment. In this respect, the ability of self medication might build an important evolutionary advantage.

To some extent fluctuations in the supply of the secondary plant compounds are tolerated by the sifakas (e.g. due to seasonal variations and different food supply), but strong alterations may lead to disease and death. From necropsy reports it can be concluded that chronic diarrhoea led to multiple generalised systemic infections and finally to a septicaemia or to a metabolic disease.

Hence, the daily ingestion of antidiarrhoeal agents with preventive and curative properties are obviously needed to maintain the difficult equilibrium between the gastrointestinal microflora, the mucosa and the host immune system. The long-term antidiarrhoeal therapy mirrors the requirements to maintain a well-functioning intestinal barrier. Inherited genetic predispositions, bacterial, viral, environmental or host factors or a combination of these may contribute to the impairment of this equilibrium. This raises the central question:
What does this equilibrium disturb? In other words, why do the sifakas actually need a long-term therapy of phytotherapeutics with antidiarrhoeal and curative effects on their gastrointestinal system to stay healthy?

Although reasons may be very complex and multifactorial, it is reasonable to presume an increased gastrointestinal permeability is a common feature in all these clinical courses. Hence three hypotheses may be formulated:

1.) Lemurs and in particular members of the indriid family, are extremely susceptible to gastrointestinal bacterial or viral infections which may lead to bacterial overgrowth and mucosal damage and as consequence may suffer from an increased gastrointestinal permeability with known development of systemic infections and septicaemia by massive bacterial translocation.
2.) The mucosal immune system is easily impaired by bacterial or viral products which in turn disturb the mucosal barrier leading to diarrhoea and ultimately to an increased gastrointestinal permeability.
3.) An increased species-specific gastrointestinal permeability is inherent to all lemur species and requires mucosal protective agents to reduce the physiologically elevated gastrointestinal permeability. This third variant is tempting as all lemur species in general, prefer a diet high in tannins (Spelman *et al.*, 1989).

Feeding trials are one possible way to assess more closely the requirements to be supplied by the food. Prepared feeding trials with pressed tablets containing either tannins (tannin albuminate) or polysaccharides (Plantaginis ovatae testa) and grass meal (as auxiliary agent) have unfortunately not been approved by the Duke Lemur Center Research Council, and remain a possibility regarding sifakas' food choice.

Following all efforts to investigate the feeding behaviour of the sifakas, with numerous chemical analyses on preferred food plants (*Rhus copallina* and *Albizia julibrissin*) and additional plant species, and following a series of pharmacological assays to assess their effects and dosages ingested, I would now recommend the sifakas be investigated by a gastroenterologist in order to target the remaining central question.

E Summary

The investigation of the phenomenon of diarrhoeal diseases in captive *Propithecus verreauxi coquereli* (Coquerel's sifakas), a highly endangered Malagasy lemur species of the indriid family, was conducted at the Duke University Lemur Center (DULC), North Carolina, USA. The Indriidae are arboreal living, mainly folivorous primates possessing a highly specialised gut system, classified as midgut-fermenters[1]. Acute and recurrent bouts of diarrhoea with lethal episodes are only described for captive conditions despite veterinary treatment and albeit no obvious detrimental or noxious food had been ingested. As health conditions had already improved significantly at the DULC with the introduction of various self-selected leaf species into their diet, prior to the onset of this study, a strong correlation between the well-being of the sifakas and special dietary needs was hypothesised.

Within this study, the full dietary needs as to the role of primary and secondary plant compounds with special respect to the selected leaf species, as well as their nutritional and energy contribution and their impact on health were investigated.

The feeding behaviour of six Coquerel's sifakas of various ages that comprised four study groups were investigated during two seasons with one observation session in May, the other in October. The focal lemurs were kept in a variety of enclosures including combinations of caged and free-ranging forested habitat enclosures. On a daily basis, the groups received a variety of fruits and vegetables, out of a possible 27 different food species, leaf-eater primate chow, and 6 to 8 different leaf species cut from trees growing proximate to the DULC. The entire food intake was based on fresh weight in relation to the parameters of each lemur group, together with the lemur's bodyweight. The weight of food intake on wild browse in the forested enclosures was estimated. By devising new methodology in determining the amount of wild browse consumed, allowed this study to include natural-like feeding conditions in the feeding assessments.

Overall, 36 ± 8 % (m/m) of the entire food intake was allotted to the portion of leaves, irrespective of the animal's age, despite different feeding and holding conditions. The absolute leaf intake decreased from spring to the autumn. *Rhus copallina* (Anacardiaceae) and *Albizia julibrissin* (Mimosaceae), were the most preferred leaf species, with a significant seasonal decrease in *A. julibrissin* leaf intake (12 % (m/m) in spring versus 4 % (m/m) in the autumn). Contrarily, *R. copallina* leaves remained highly preferred with 16 ± 5 % (m/m).

The food composition in primary food components revealed a strong dependency on the animal's age in respect to nutrient and energy intake per kg bodyweight despite large variations in the selected food resources and seasonal fluctuations in all primary leaf compounds as analysed for the browse species. Both groups with juvenile and subadult animals consumed nearly double the amount of proteins and available carbohydrates per kg bodyweight than the adult groups, while the portion of fat reflected more accurately the food resources consumed than actual dietary needs. On the other hand, the respective portions (weight %) of the nutritional composition within the diets remained rather constant irrespective of the animal's age and food selection.

Different models of energy calculations showed a relatively low energy contribution by the leaves to the overall diet. Although the leaves proved to be a valuable source of proteins, dietary fibres and minerals, there was a superabundance of other food resources that could easily replace the portion of leaves to supply nutrients and energy. Therefore, the role of the

[1] previously known as hindgut-fermenting primates

secondary leaf compounds should be elucidated with respect to their impact on leaf choice and possible beneficial pharmacological effects for the sifakas' well-being.

In reflection to the observed feeding behaviour, a "classification system" was instigated to survey the occurrence of secondary plant compounds in all plant species and plant parts within their environment. Food preferences and avoidance were found to correlate with the occurrence of secondary plant compound groups and proved to be chemotaxonomically consistent. High levels of hydrolysable tannins were clearly sought out with a discrimination between hydrolysable and condensed tannins. The predilection for tannins agrees with previous observations on other lemur species described in literature.

Preparative isolations and subsequent structure elucidation by means of HPLC analyses, UV-spectroscopy, mass spectrometry and NMR-spectroscopy, elucidated unambigously the structure of four flavonoid glycosides in *R. copallina* leaves including a new auronol, and six flavonoid glycosides in *A. julibrissin* leaves, with only three to be expected in *A. julibrissin* as described in the literature. Two flavonoid glycosides were found to occur in both plant species. One flavonoid glycoside in *A. julibrissin* leaves was found to be novel, quercetin-3-O-α-L-rhamnopyranosyl(1$\rightarrow$2)ß-D-xylopyranoside. Additional eight flavonoid glycosides were proposed in structural constituents and molecular weight for *R. copallina* leaves, and nine for *A. julibrissin* leaves, with acylated glycosides within both species appearing as a new feature.

A total of 11 gallic acid derived compounds were identified within *R. copallina* leaves including a new isomeric p- and m-digallic acid methyl ester, while four simple phenolic compounds were identified within *A. julibrissin* leaves, with two compounds being in common within both leaf species. Only in part were these compounds expected from literature.

Quantification by HPLC methods revealed gallotannins to be the major compounds within *R. copallina* leaves, and quercetin derived flavonol glycosides within *A. julibrissin* leaves, that were also found to occur within additionally selected leaf species. In agreement with the current literature, the highest contents of hydrolysable tannins were found within *R. copallina* leaves showing a seasonal decrease from approximately 28 % (m/m) in May leaves to 18 % (m/m) in October leaves, followed by *Acer rubrum* with approximately 18 % (m/m) in May leaves and 12 % (m/m) in October leaves. Highest yields of flavonol glycosides were analysed for *A. julibrissin* in the May leaves with 4.85 % (m/m) with decreasing levels in the October leaves. Only the younger October leaves were ingested by the animals with lower quercitrin levels (approximately 85 % relative abundance of quercitrin in older October leaves versus 58 % in the younger leaves) and overall higher flavonoid contents.

A pectic-like mucilage detected in *A. julibrissin* leaves was found to consist of rhamnose, galactose and galacturonic acid in nearly equal molar ratios and a molecular weight of approximately 2 million Da. This rhamnogalacturonan was characterised for the first time in *A. julibrissin* leaves and was the third major leaf compound ingested by the sifakas. Linkage analyses, including various mass spectrometric experiments and partial hydrolysis, yielded tetramerous, hexamerous and octamerous linear chains, for which a backbone structure with alternating 1,2-linked rhamnose and 1,4-linked galacturonic acid residues was proposed. In branched regions that also contain galactose residues as the third major component, 1,2,4-linked rhamnose appeared as the site of ramification. This proposed structure was found to be in agreement with hairy regions in pectins and pectin-like polysaccharides.

The leaf mucilage proved compatible with gallotannins, which is undoubtedly important as *A. julibrissin* and *R. copallina* leaves were consumed within one meal time. A swelling number (QZ) of 9 was determined for those *A. julibrissin* leaf harvests, which were utilised for subsequent dosage calculations.

To evaluate the pharmacological relevance of the three major leaf compounds in the lemur diet with respect to preventive and curative effects in diarrhoeal diseases, different antimicrobial *in vitro* assays were performed that targeted possible antibacterial, antifungal and antiprotozoal effects. However, various *R. copallina* leaf extracts showed best growth-inhibitory effects against several *Staphylococcus aureus* strains, against *Pseudomonas aeruginosa*, *Helicobacter pylori*, two species of *Corynebacterium* and *Bacteroides fragilis*, while no antifungal or antiprotozoal activity was found. *A. julibrissin* leaf extracts showed no activity. Furthermore, in a gel-electrophoretic binding assay, tannin extracts from *R. copallina* leaves inactivated *Cholera* toxin by directly interacting with the A subunit of the toxin in a dose-dependent manner. Seasonal influences appeared to impact the binding affinities of the leaf extracts to the toxin.

Evidence for antidiarrhoeal, protective and normalising effects onto processes within the gastrointestinal tract was extracted from the literature for all three major groups of leaf compounds, showing each a large therapeutic broadness. To ultimately evaluate the relevance of these secondary compounds ingested by the sifakas with the daily leaf intake, averaged ingested dosages were calculated and compared to therapeutic dosages as utilised within human medicine. It became clear that all dosages ingested by the sifakas lay within the therapeutic dosage range as recommended for humans. While overall the consumed secondary compounds decreased parallel to the seasonal decrease in leaf contents and leaf intake; the animal's age did not appear to influence dosage intakes.

The three major groups of secondary plant compounds selected by the sifakas are known to occur ubiquitously in plant-kingdom, and their phytotherapeutic utilisation may be conceived as a long-term triple therapy. Deprivation of these chemical compounds may risk illness and death, an outcome of an animal type that possesses a highly specialised gut system that is adapted to special dietary needs.

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Appendix I
Observation and quantification of the food composition

Table of contents

I.1 Scoring the first ten different food species eaten by each individual sifaka

The first ten different food species selected by each focal animal correspond to ranks (numbering from 1 to 10), which were recorded when a plant species was changed during the main feeding bout inside the cage. The number given per rank in Tab. I.1 is the sum of frequencies over all study days when an individual selected different food species. These ranks do not infer the amount ingested, nor the time spent feeding on any food type.

Tab. I.1: Tables a-d summarise the frequencies of the first ten different species selected by each individual sifaka. Except for group J&D, who was only fed once a day, ranks of the second main feeding bout in the evening, are depicted here.

a) Group M&T, who was observed for 17 days:

rank	Marcella										Tiberius									
leaves	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
R. copallina	5	8	4	10	6	6	5	7	4	7	6	5	7	8	5	7	6	4	5	3
A. julibrissin	11	6	9	5	6	9	4	5	6	8	11	5	9	6	10	6	3	7	4	5
A. rubrum	1	3	2	2	1	1	3	2	4			4		1		2	1	1		2
C. canadensis							2	1	1			1			1		1		1	1
L. styraciflua			1		1			1				1								
R. pseudoacacia			1		1		1							1						
P. glabra					not fed										not fed					
L. tulipifera																				
Mango fruit and leaves																	1		1	
chow					2		1	1				1		1		1	1	3	1	1
fruits&veg.						1	1		2	1			1		1		3	1	3	3

b) Group D&V, who was observed for 6 days:

rank / leaves	Drusilla										Valentinian									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
R. coapllina	1	2	3	2	3	1	2	1	2	1	1	1	1	2	2	2	1	2	2	3
A. julibrissin	4		2	3	3	2	2	3	2	3	3	2	2	2	4	2	4	1	2	
A. rubrum		3	1			1	2	1		1			1	1					1	1
C. canadensis	1	1				1			1		2	3	1	1		1	1	1	1	1
L. styraciflua				1		1		1					1			1		1		
R. pseudoacacia																		1		1
P. glabra					not fed										not fed					
L. tulipifera					not fed										not fed					
Mango fruit and leaves					not fed										not fed					
chow																				
fruits&veg.																				

c) Group J&D, who was observed for 11 days:

rank / leaves	Julian										Drusilla									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
R. copallina			3	2	1	5	3	1	3	3					1		1	1		4
A. julibrissin	3	1		1	3	2	2	3		1	1	1	3		2		2			
A. rubrum					not fed										not fed					
C. canadensis																				
L. styraciflua																				
R. pseudoacacia																				
P. glabra									1											
L. tulipifera						1	1													
Mango fruit and leaves					not fed										not fed					
chow	1	2			1	1	1	1	4									3		2
fruits&veg.	7	8	8	8	6	2	3	5	2	4	10	10	8	11	8	11	8	7	11	5

d) Gordian, who was observed for 11 days:

rank / leaves	Gordian									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
R. copallina	9	2	9	1	8	2	6	2	5	1
A. julibrissin		5	2	6	2	6	2	4	1	4
A. rubrum					not fed					
C. canadensis	1									
L. styraciflua				1					1	1
R. pseudoacacia		1						1		
P. glabra		1				1		1		1
L. tulipifera	1	2		3		1	1			1
Mango fruit and leaves					not fed					
V. rotundifolia[a]										
chow									1	
fruits&veg.										

[a] Some *Vitis rotundifolia* was embracing black locust and given that way to Gordian.

I.2 Feeding pattern of Marcella and Tiberius in the forest

The amount of food consumed in the forest (estimated in g, see B.2.4) and the time spent feeding are differentiated for both Marcella and Tiberius. “Total” designates the total weight ingested while feeding in the forest as estimated for both animals; “time outside” designates the total hours spent in the forest. The percentages give the portion of time spent feeding related to the time spent in the forest.

Tab. I.2: Weight [g] ingested and time spent feeding in relation to the time spent daily in the forest, respectively for Marcella and Tiberius.

	Marcella			**Tiberius**			**total**	
date	**estimated weight [g]**	**time spent feeding [min.]**	**% of time outside**	**estimated weight [g]**	**time spent feeding [min.]**	**% of time outside**	**estimated weight [g]**	**time outside [h]**
17.5.	14.0	12	5.7	9.1	13	6.2	23.1	3.5
18.5.	21.0	33	14.5	12.8	37	16.2	33.8	3.8
19.5.	9.6	12	6.7	4.2	8	4.4	13.8	3.0
20.5.	27.6	39	32.5	15.1	36	30.0	42.7	2.0
21.5.	48.7	28	11.7	35.3	26	10.8	84.0	4.0
22.5.	9.9	16	10.7	19.3	24	16.0	29.2	2.5

I.3 Taste discrimination of plant leaves from foliage included in spring browse

R. copallina: absolutely astringent, like black tea brewed too long
A. julibrissin: pleasantly slimy, followed by bitter note
L. styraciflua: extremely sour
C. canadensis: bitter and astringent
A. rubrum: extremely bitter and astringent
R. pseudoacacia: sticky, slimy, and bitter; a mild astringent taste

R. copallina and *A. julibrissin* exhibited the most distinctive tastes (to me) compared to their intrinsic chemical compounds that were analysed in this study.

Appendix II
Chemical investigations on primary and secondary plant compounds

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II.1 Nutritional values

Tab. II.1: Nutritional values of the leaves in gram per kg air-dried material according to Weender analyses. Except for the contents of starch and simple saccharides duplicate analyses were performed.

species		dry substance	crude protein	starch[a]	simple saccharides[a]	crude fibre	crude ash
A. julibrissin	May	897.5	305.0	41.3	38.3	100.9	51.6
A. julibrissin	July	908.8	253.4	21.8	27.4	134.7	60.4
A. julibrissin	Oct., y.l.	907.6	195.3	21.8	39.2	149.3	58.2
A. julibrissin	Oct., o.l.	911.0	229.3	26.0	48.3	136.5	52.0
R. copallina	May	849.7	147.7	10.8	45.6	74.3	29.5
R. copallina	July	914.8	118.9	24.0	71.9	109.3	28.3
R. copallina	Oct.	915.7	105.1	21.8	100.3	128.8	27.9
A. rubrum	May	922.4					33.7
A. rubrum	Oct.	925.8					44.6
R. pseudoacacia	May	908.0					56.6
R. pseudoacacia	Oct.	917.9					56.6
L. styraciflua	May	884.4					39.8
L. styraciflua	Oct.	915.3					70.1
C. canadensis	May	912.4					50.7
C. canadensis	Oct.	909.7					67.6
L. tulipifera	Oct.	911.5					65.7
P. glabra	Oct.	903.8					51.9
V. rotundifolia	Oct.	899.1					39.7
chow		911.7					64.1

[a] simple analyses were performed due to low plant material

II.2 Literature review on secondary plant compounds

Tab. II.2**:** Review on secondary plant compounds occurring in plant species of the animals' environment including known pharmacological effects, and assignment of the animals' behaviour towards these plants / individual plant parts, (called "classification"; see B.3.2). Alphabetical order is based on plant families, of featured species.

family, genus, species and plant part	classification
Aceraceae (order: Sapindales) Maple syrup is gained from *Acer saccharum*, *A. saccharinum* and *A. rubrum*. In Aceraceae the ureides allantoin and allantoic acid play an important role in nitrogen metabolism.	
Acer Condensed and hydrolysable tannins (gallo- and ellagitannins), both occur in varying amounts depending on *Acer* species (Bate-Smith, 1962; 1977); especially acertannin (2,6-di-O-galloyl-1,5-anhydro-D-sorbit) occurs; quebrachit (a cyclitol) abundant; quercetin and kaempferol glycosides are abundant in leaves. Other phenolic compounds are sporadically appearing, simple phenolics are common. Saponins occur in leaves and barks of some species. The bark may contain quinones with anti-inflammatory effects (Hegnauer, 2001).	
Acer rubrum L. <u>commercial use</u>: Pioneers made ink, cinnamon-brown and black dyes from bark extracts (Little, 1993). <u>medicinal use</u>: A decoction of *Acer rubrum* (bark) was used by the Cherokees for sore eyes. Further indications were linked to its astringency: fever, diarrhoea and gynaecological disorders (Crellin and Philpott, 1990).	
leaves <u>alkaloids</u>: gramine (trace amounts) (Barbosa *et al.*, 1990); <u>saponins</u> (Hegnauer, 2001); <u>tannins</u>: predominance of gallo- and ellagitannins (Bate-Smith, 1978); acertannin and pentagalloylglucose (Hegnauer, 2001)	eaten
fruits <u>alkaloids</u>: gramine (0.03 % of the dried fruit) (Hegnauer, 2001)	
bark <u>condensed tannins</u>: catechin units (Hegnauer, 2001); procyanidin trimer (Narayanan and Seshadri, 1969)	
wood <u>sterols</u>: ß-sitosterol (Narayanan and Seshadri, 1969); <u>condensed tannins</u> (Narayanan and Seshadri, 1969)	
Anacardiaceae (order: Rutales) Anacardiaceae are rich in tannins: gallo- and ellagitannins predominantly in leaves, barks and galls [→ nutgalls from various *Rhus* species (Chinese Gallae) contain pure gallotannins (Frohne and Jensen, 1992)]; condensed tannins predominantly in woods (heartwood: up to 15-25 %): fisetin (5-deoxyflavonol), fustin (5-deoxyflavanonol) and leucofisetinidin (5-deoxyflavan-3,4-diol) as units, aurones and auronols (Hegnauer, 2001). Biflavonoids occur. Myricetin, quercetin and kaempferol glycosides are abundant, apigenin and luteolin glycosides are sometimes in leaves. Different types of excretion organs contain: oleoresins, oleoresins plus mucilaginous compounds, or latex. In genera *Rhus* and *Toxicodendron* latex occurs with phenolic compounds causing contact dermatitis (Hegnauer, 2001; Frohne and Jensen, 1992). Organic acids (quinic acid and shikimi acid) are abundant in leaves.	

family, genus, species and plant part	classification
Mangifera indica L.	
drugs: Mango-fruits (rich in ß-caroten) (Frohne and Jensen, 1992).	
Silica abundant in leaves (Mangiferae). Triterpenoids occur in resins and leaf galls. Latex contains skin irritating alkenylphenolic compounds (alkenylresorcinols), (Hegnauer, 2001).	
leaves	tasted
essential oils: 0.013 % (Hegnauer, 2001);	
flavonoids: quercetin, kaempferol glycosides (Hegnauer, 2001; ElSissi *et al.*, 1971);	
tannins: condensed tannins (catechin, epicatechin-3-gallat units); gallo- and ellagitannins (ElSissi *et al.*, 1971; Hegnauer, 2001);	
xanthones and associated compounds:	
7.1 % mangiferin (1,3,6,7-tetrahydroxyxanthone-2-glucopyranoside); galloyl derivatives and 4-hydroxybenzoyl derivatives of mangiferin; 3-C-glucomaclurine (benzophenone), 3-C-glucoiriflophenone; isomangiferin; protocatechuic acid (ElSissi *et al.*, 1971; Hegnauer, 2001)	
flowers	
essential oils: 0.04 %;	
gallic acid derivatives: gallic acid ethyl ester (Hegnauer, 2001)	
fruits	eaten
carotenoids (Hegnauer, 2001)	
bark	
tannins: 10-20 %;	
xanthones: mangiferin and -3-methylether (homomangiferin) (Hegnauer, 2001)	
leaves, bark (see above), wood and fruit	
xanthone: mangiferin (Hegnauer, 2001)	
flowers, fruits, stems	
phenolic phytoalexines (Hegnauer, 2001)	
seeds	
tannins: 8-9 % (Hegnauer, 2001)	
Rhus	
Hydrolysable (gallo-)tannins are abundant in leaves and barks of all *Rhus* species: contents up to 40 % (referred to dry leaf weight).	
Chinese Gallae (of *Rhus semialata*): up to 78 % tannins.	
Investigation of eight North American *Rhus* species revealed high tannin contents in leaves and flowers, while stems, barks, woods and seeds exhibited relatively low contents.	
Leaflets: high tannin contents; rachis and leafstalks: relatively low tannin contents;	
Heartwood of *Rhus* species: condensed tannins (see above) (Hegnauer, 2001).	
drugs and medicinal use: According to U.S. Pharmacopoeia, tannic acid is obtained from “nutgalls or leaves of sumac” (any *Rhus* species). So, commercial tannins are gained either from Chinese Gallae (from various *Rhus* species, like *R. semialata* or *R. sinensis*), or from leaves of *R. coriararia* (Doorenbos and Box, 1976; Frohne and Jensen, 1992). (For tannins gained from Turkish galls, see *Quercus*).	
Medicinally, tannin is used as antidiarrhoeal: tannin protein complex (ratio 1:1) (tannin albuminate, Albumini tannas).	
leaves	
flavonoids: myricetin, quercetin and kaempferol glycosides abundant	
fruits	
organic acids: predominantly malic acid (6.5-18 %);	
anthocyanins (Hegnauer, 2001)	

family, genus, species and plant part	classification
Rhus copallina L.	
use: The sour fruit can be nibbled or made into a drink like lemonade. Wildlife is known to eat the fruit, deer browse on the twigs (Little, 1993). Due to high tannin contents of leaves and berries, sumac was recommended as substitute for galls in dying, the manufacture of ink and tanning (Crellin and Philpott, 1990).	
medicinal use: *Rhus* species is a source of USP quality tannin: see above. According to Crellin and Philpott (1990), *Rhus coriararia*, *R. glabra* and *R. copallina* were used the same way in domestic medicine (the vernacular name “red sumac” covers the species *R. glabra* and *R. copallina*, while “common sumach” refers to *R. coriararia* and *R. glabra*). Usage of leaves, roots and berries is mainly based on their astringent properties. Hence, sumac species were used for sore throats and mouths, dysentery, diarrhoea, burns and haemorrhoids. Indians used the leaves externally for sores and swellings. Due to its content of vitamin C and malic acid, fruits were used as cooling medicine for fevers and as cough medicine. Currently, leaves and berries are recommended as antidote for poisons (Crellin and Philpott, 1990).	
leaves	eaten
tannins: three geographic races can be discerned with respect to leaf tannin contents (referred to dry matter): eastern race: 27 %; southern race: 33 %; central race: 36 % (Hegnauer, 2001)	
remark: According to Doorenbos and Box (1976) efforts were made to use *R. copallina* and. *R. glabra* as native sources of commercial gallotannins. Starting in 1965, tannin content of the dried leaves of individual trees varied enormously from 4-38 %. Therefore, only plants of clones yielding at least 30 % tannins (referred to dry leaf weight) were used for cultivation trials.	
Rhus radicans L.	untouched
The latex contains urushiols (3-alkyl- and 3-alkenyl-brenzcatechols) causing contact dermatitis (poison ivy dermatitis, *Rhus* dermatitis) (Hegnauer, 2001; Frohne and Jensen, 1992).	
medicinal use: In homeopathy fresh roots are used e.g. for skin irritations, neuralgia and colds (HAB; Hagers Handbuch, 1994).	
Betulaceae (order: Fagales)	
Accumulation of tannins in barks and leaves; the occurrence of condensed tannins (leucocyanidin units), hydrolysable (gallo- and ellagi-)tannins and hydroxycinnamic acids varies with genera and species. triterpenoids: pentacyclic in barks, and penta- and tetracyclic in leaves; flavonoids: mainly hyperosid or myricitrin as glycosides in leaves (Frohne and Jensen, 1992; Hegnauer, 2001). (see also Fagaceae)	
Carpinus	
leaves	
flavonoids: myricetin, quercetin, kaempferol glycosides (Hegnauer, 2001); tannins: gallotannins (?) (Hegnauer, 2001); hydroxycinnamic acids: chlorogenic acid (Hegnauer, 2001)	
Carpinus caroliniana Walt.	
medicinal use: Bark, wood and leaves were used as tonic and for gastric disorders. Indians used it in preparations for coughs, haemorrhages, kidney problems, and rheumatism (Crellin and Philpott, 1990).	
leaves	tasted
flavonoids: hyperoside and other quercetin glycosides (Hegnauer, 2001)	

family, genus, species and plant part	classification
Caesalpiniaceae (order: Fabales)	
drugs (selection):	
Used as laxatives: Sennae folium et fructus (*Cassia senna* and *C. angustifolia*). Used as laxative and in the therapy of biliary disorders: Tamarindi pulpa from fruits of *Tamarindus indica* (organic acids and a heteropolysaccharide (a galactoxyloglucan with gelling properties), (Frohne and Jensen, 1992; Hegnauer, XIa, 1994; Jänicke *at al.*, 2003).	
Gummosis in Caesalpiniaceae species is more seldom than in Mimosaceae, but occurs (Hegnauer, XIa, 1994).	
Cercis	
leaves	
tannins: condensed tannins (Hegnauer, XIa, 1994);	
flavonoids: myricitrin (Hegnauer, XIa, 1994), monoglycosides of quercetin, myricetin and kaempferol (Hegnauer, XIb-2, 2001)	
flowers	
flavonoids: myricitrin (Hegnauer, XIa, 1994)	
Cercis canadensis L.	
use: The flowers can be eaten as salad, or fried (Little, 1993).	
medicinal use: The bark has been used as an astringent, for the treatment of diarrhoea and dysentery, especially when it becomes chronic (Crellin and Philpott, 1990).	
leaves and pods	eaten
Caprifoliaceae (order: Dipsacales)	
Iridoids and secoiridoids abundant in the family, partly as complex esterglycosides; loganin (iridoid) in *Lonicera* and *Viburnum*;	
caffeic acid as common characteristic (mainly as chlorogenic acid); mono- and dicaffeoyl-quinic acid abundant in leaves; saponins abundant; free ursolic acid may occur in leaves; the occurrence of flavonoid glycosides and coumarins is characteristic of individual genera (Frohne and Jensen, 1992; Hegnauer, 2001).	
Lonicera	
Iridoid alkaloids (pseudoalkaloids) occur in *Lonicera.*	
leaves	
hydroxycinnamic acids: chlorogenic, caffeic, p-coumaric acid;	
flavonoids: glycosides of apigenin and luteolin predominate, but quercetin and kaempferol glycosides also occur (Hegnauer, 2001)	
bark	
syringin or coniferin depending on species (Hegnauer, 2001)	
Lonicera japonica Thunb.	
use: Sweet nectar can be milked from the base of the corolla (Niering and Olmstead, 1992).	
flowers	tasted
leaves	
flavonoids: 0.01 % lonicerin (= luteolin-7-rhamnoglucosid) in fresh leaves;	
saponins (Hegnauer, 2001)	
fresh new twigs	
iridoid compounds: 0.5 % secoxyloganin (Hegnauer, 2001)	
Viburnum	
Storage of dihydrochalcones (Hegnauer, 2001).	
leaves	
hydroxycinnamic acids: chlorogenic, isochlorogenic, neochlorogenic acid;	
flavonoids: quercetin and kaempferol glycosides abundant (Hegnauer, 2001)	
fruits	
anthocyanins: cyanidin-glycosides (Hegnauer, 2001)	
bark	
condensed tannins: (catechin, epicatechin units) (Hegnauer, 2001)	

family, genus, species and plant part	classification
Viburnum prunifolium L.	untouched
drug: Viburni prunifolii cortex: the astringent bark was used medicinally; officinal drug in USA and some European countries (Hegnauer, 2001).	
bark	
Viburni prunifolii cortex (see above);	
flavonoids, coumarins and other phenolics: salicin (glucoside of o-hydroxybenzylalcohol); amentoflavone (biflavone); scopoletin (coumarin); 0.01 % triester of 1,2,3-benzenetricarbonic acid (= 1-methyl-2,3-dibutylhemimellilat), (Hegnauer, 2001)	
bark and leaves	
phenolic glycosides: arbutin (Hegnauer, 2001)	
bark, leaves and flowers	
triterpenoids: of α- and ß-amyrin type (Hegnauer, 2001)	
root bark	
iridoid glycosides (Hegnauer, 2001)	
Viburnum rafinesquianum	
leaves	refused
Cornaceae (order: Cornales)	
Iridoid and secoiridoid compounds are characteristic of the family. Galli- and ellagitannins are abundant (Tanaka *et al.*, 2001). Ellagitannins: cornusiin-A and -B. Triterpenoids and triperpenoid saponins are abundant (Frohne and Jensen, 1992; Hegnauer, 2001).	
Cornus	
leaves	
flavonoids: quercetin and kaempferol glycosides abundant;	
tannins: gallo- and ellagitannins, condensed tannins;	
hydroxycinnamic acids: caffeic acid abundant;	
phenolic glucosides: 0.5-1.2 % cornus-quinol-glucoside (= cornoside) and salidroside (p-hydroxy-phenyl-ethanol-glucoside) (Hegnauer, 2001)	
Cornus florida L.	
medicinal and other use: Indians used aromatic barks and roots as remedy for malaria and extracted a red dye from roots (Little, 1993). Tree bark and root bark were included in the USP as cinchona substitute for fevers and bitter tonic / stomachicum (Crellin and Philpott, 1990).	
leaves	refused
anthocyanins: cyanidin-3-glucosid;	
iridoid compounds: cornin and dihydrocornin;	
cyclitols (inositol);	
triterpenoids: 0.8-1 % free ursolic acid (Hegnauer, 2001);	
saponins with molluscicidal properties (Barbosa *et al.*, 1990)	
flowers	
flavonoids: quercetin, kaempferol glycosides;	
tannins: gallotannins;	
triterpenoids (ursolic acid, nonacosan, betulininic acid);	
cyclitol (scyllitol, inositol) (Hegnauer, 2001)	
fruits	
anthocyanins (Hegnauer, 2001)	
bark	
steroid saponin glycosides: filiferin, sarsapogenolglycosides (Hegnauer, 2001)	
bark and root bark	
iridoid compounds: 4 % cornin (= verbenalin; bitter tasting compound) in the bark (Frohne and Jensen, 1992; Hegnauer, 2001)	

family, genus, species and plant part	classification
Ebenaceae (order: Ebenales)	
Tannins and naphthoquinone derivatives are characteristic of the family and abundant in *Diospyros*, especially in the heartwood (e.g. ebony of *D. ebenum*) (Frohne and Jensen, 1992; Hegnauer, 2001); but also in other organs like barks, roots, fruits and leaves. Oxidation products of 1,8-dihydroxynaphthaline and corresponding naphthoquinones cause brown and black pigments found so far, especially in decomposing plant organs. Methyljuglones (like plumbagin) build basic structures, while also di-, tri- and tetrameric naphthoquinones occur (Hegnauer, 2001).	
Pharmacological effects: ichthyotoxic, insecticidal and anthelminthic; irritations on skin contact; resistance against termites.	
Flavonol glycosides (quercetin-3-glycosides) occur in leaves.	
Condensed tannins (catechin, leucocyanidin and leucodelphinidin units, in part esterified with gallic acid) predominate in *Diospyros* species (especially in barks and fruits, partly also in leaves), but gallotannins and coumarins are also abundant. Naphthoquinone-5-methylcoumarin dimers and trimers, anthraquinone glycosides, lignans and ellagic acid derivatives also occur.	
Accumulation of free pentacyclic triterpenoids in the family (e.g. oleanolic acid, ursolic acid, lupeol, betulin and betulinic acid) in different organs like roots, barks, woods, leaves, flowers, fruits and seeds. Triterpenoid saponins occur (e.g. in leaves and fruits) which build together with caustic quinones the anthelminthic and ichthyotoxic principle of these plants. ß-sitosterol abundant. Alkaloids (of unknown structure) may occur in leaves and barks of *Diospyros* species (Hegnauer, 2001).	
Diospyros virginiana L.	
use: Persimmons are consumed fresh and used to make puddings, cakes, and beverages. American Indians made persimmon bread and stored the dried fruit like prunes. *Diospyros* (Greek) means "fruit of the god Zeus" (Little, 1993).	
medicinal use: The bark, especially the inner bark and unripe fruit were used as astringent, tonic, for fevers (like cinchona), and as antiseptic. The unripe fruit was recommended for diarrhoea, gastrointestinal disorders and haemorrhage due to its content of tannins. Up to 1880 persimmon was included in the U.S. Pharmacopoeia (Crellin and Philpott, 1990).	
leaves	tasted in spring; eaten in the autumn
flavonoids: myricetin, quercetin and kaempferol glycosides (Hegnauer, 2001);	
tannins: condensed tannins (leucodelphinidin units) (Hegnauer, 2001);	
hydroxycinnamic acids: p-coumaric acid (Hegnauer, 2001);	
organic acids: leaves are rich in ascorbic acid (Hegnauer, 2001)	
fruits	
tannins: condensed tannins (Hegnauer, 2001): up to 18 % in the unripe fruit (referred to dry weight) (Crellin and Philpott, 1990)	
bark and wood	
naphthoquinones: isodiospyrin (Hegnauer, 2001)	
Ericaceae (order: Ericales)	
Ericaceae are characterised by polyphenolic compounds of different types: phenolic glycosides, flavonoids and / or tannins (condensed and / or hydrolysable (ellagi-) tannins) (Frohne and Jensen, 1992). Kaempferol and especially quercetin abundant. Sometimes myricetin is found.	
Different hydroxycinnamic acids (p-coumaric acid, caffeic acid etc.) sporadically. Arbutin (a hydroquinone-glucoside, e.g. as antibacterial principle in Uvae ursi folium) abundant in some species.	
Some species accumulate essential oils (in glandular hairs) (Hegnauer, 2001). Toxic diterpenes occur in leaves, twigs, wood, flowers and even in nectar ("toxic honey") of some genera (Hegnauer, 2001).	
Iridoid compounds (C_{10} and C_9 type) occur in different genera and species. Cuticle wax of leaves is rich in triterpenoids, especially in ursolic acid, but also other triterpenoid compounds (like saponins) occur in different organs (Frohne and Jensen, 1992; Hegnauer, 2001).	
Organic acids occur widely in leaves and fruits (Hegnauer, 2001).	

family, genus, species and plant part	classification
drugs: Myrtilli fructus et folium (*Vaccinium myrtillus*) (predominantly condensed tannins) are used as antidiarrhoeal agent (Jänicke *et al.*, 2003).	
Oxydendrum	
leaves and bark	
tannins (Hegnauer, 2001)	
Oxydendrum arboreum (L.) DC.	
use: Sourwood honey is esteemed (Little, 1993).	
medicinal use: Leaves are said to be tonic, refrigerant and diuretic. In domestic practice leaves are used in form of infusion or decoction as refrigerant drink for fevers (Crellin and Philpott, 1990).	
leaves	tasted
hydroxycinnamic acids: leaves are rich in caffeic acid (Hegnauer, 2001);	
no toxic diterpenes (of andromedotoxin-type) (Hegnauer, 2001);	
organic acids: sour taste of the foliage (Little, 1993; Crellin and Philpott, 1990)	
bark	eaten
Fabaceae (order: Fabales)	
Alkaloids occur in the family, but are normally restricted to special tribes or occur sporadically.	
Saponins (triterpenoid saponins e.g. in *Glycyrrhiza glabra*; steroid saponins e.g. in *Trigonella foenum-graecum*) are abundant.	
Isoflavonoids (with oestrogen-like effects) and rotenoids (e.g. ichthyotoxic and insecticidal) have several pharmacological and ecological effects (functioning as phytoalexins, fungitoxic), (characteristic).	
Cyanogenic compounds are frequently found (Frohne and Jensen, 1992).	
Gummosis rarely occurs (Hegnauer, XIa, 1994).	
drugs (selection):	
Oleum Arachidis (*Arachis hypogaea*); Tragacantha (Tragant, Ph.Eur.; an arabinogalactan), the dried exudate from trunk and twigs of *Astragalus* species (e.g. *A. gummifer*), is used as laxative and as stabiliser in the pharmaceutical technology and food chemistry. Guar galactomannanum (Guargalactomannan, Ph.Eur.) is gained from the ground endosperm of seeds of *Cyamopsis tetragonoloba*, which is used in the therapy of diabetes and lipaemia, and like the former polysaccharide as laxative and as stabiliser in the pharmaceutical technology and food chemistry.	
Further drugs: Balsamum peruvianum (*Myroxylon balsamum*); Liquiritiae radix et succus (*Glycyrrhiza glabra*); Meliloti herba (different *Melilotus* species); Ononidis herba (*Ononis spinosa*); physostigmin (*Physostigma venenosum*) (Frohne and Jensen, 1992; Rimpler, 1999).	
Robinia pseudoacacia L.	
officinal drug: Robiniae pseudoacaciae flos, (Wagner, 1999; Madaus, 1976). which were used as anti-spasmodic. Large doses have emetic and purgative effects.	
Compounds within the leaves provoke fever, oedema and obstipation. In homeopathy the fresh barks and young twigs are used e.g. against gastric disorders, ulcers, obstipation and headache (HAB). Notes on plant parts used are not consistent; for a review see Madaus (1976).	
plant part not specified	
flavonoids: kaempferol-3-O-rhamnosyl-galactoside 7-O-rhamnoside; luteolin-7-O-glucoside; acacetin-7-O-glucoside (Kaneta *et al.,* 1980; Hegnauer, XIa, 1994); robinin (anti-inflammatory activity) (Lipkan *et al.*, 1981)	
leaves	eaten
tannins: 8.2 % (Hegnauer, XIb-2, 2001); condensed tannins (leucodelphinidin and leucocyanidin units, and two unidentified compounds) (Kumar and Horigome, 1986); prodelphinidin more than procyanidin (Hegnauer, XIa, 1994)	
flavonoids: 1.7 % acacetin-7-glycosides (biosides and triosides, among the latter linarin); apigenin-7-bioside and –trioside (Hegnauer, XIb-2, 2001)	
rare non-protein amino acids (young leaves): canavanin (toxic), ethanolamine (Obatake and Suzuki, 1985)	

family, genus, species and plant part	classification
flowers	
<u>flavonoids</u>: 4-5 % kaempferol-3-robinobioside[1]-7-rhamnoside (robinin) with diuretic properties (Hegnauer, XIb-2, 2001)	
<u>essential oils</u>	
<u>sterols in the pollen</u>: catasterone, 6-deoxycatasterone, typhasterol (Abe *et al.*, 1995)	
fruits (unripe)	
<u>flavonoids</u>: quercetin- and kaempferol-3-robinobiosides (Hegnauer, XIb-2, 2001)	
<u>non-protein amino acids</u>: DOPA (3,4-dihydroxyphenylalanin, toxic); albizziin (Hegnauer, XIa, 1994)	
bark	
<u>tannins</u>: 7 % (Hegnauer, XIa, 1994); 2.8 % (Hegnauer, XIb-2, 2001);	
two <u>lectins</u>, called RpbAI and RPbAII from the inner bark (= *Robinia pseudoacacia* bark agglutinins I and II) (Van Damme *et al.*, 1995);	
<u>saponins</u>	
<u>toxic albumins</u> (robin and phasin)	
heartwood	
<u>condensed tannins (monomeric units and biflavanoids)</u>:	
leucorobinetinidin (flavan-3,4-diol; 3, 3', 4, 4', 5', 7-hexahydroxoflavan), 2.3 %;	
dihydrorobinetin (flavanonol; 3, 3',4', 5', 7-pentahydroxoflavan-4-on), 20 %;	
two isomeric robinetinidol-(4ß,6)-leucorobinetinidin (with 4''α- and ß-hydroxyl group, resp.);	
two isomeric robinetinidol-(4α, 2')-leucorobinetinidin (with 4''α- and ß-hydroxyl group, resp.);	
robinetinidol-(4α, 2')-dihydrorobinetin;	
robinetinidol-(4ß,6)-dihydrorobinetin;	
robinetinidol-(4ß,8)-dihydrorobinetin;	
robinetinidol-(4α, 2')-robinetin;	
robinetinidol-(4ß, 2')-tetrahydroxy-flavone (Coetzee *et al.*, 1995; Hegnauer, XIb-2, 2001);	
robinetinidol (flavan-3-ol), trace amounts (Hegnauer, XIb-2, 2001);	
<u>associated flavonoids</u>: 1.4 % robinetin (flavonol), 0.5 % robtin (flavanon), trace amounts of 5-deoxyflavonoids (fisetin, fustin, butin) and chalcones (Hegnauer, XIb-2, 2001)	
wood	
<u>tannins</u>: 3 % (Hegnauer, XIa, 1994)	
seeds	
<u>lectins</u>: robin (toxic) (Frohne and Jensen, 1992);	
<u>rare non-protein amino acids</u>: canavanin (toxic) (Frohne and Jensen, 1992; Hegnauer, XIb-2, 2001)	
pods and seeds	
<u>benzoquinone</u>: 2,6-dimethoxybenzoquinone (antibiotic), (Hegnauer, XIb-2, 2001)	
Fagaceae (order: Fagales*)*	
The order Fagales is rich in calcium oxalate, polyphenols and tannins (characteristics). Triterpenoids are abundant.	
Both, condensed and hydrolysable (gallo- and ellagi-) tannins are abundant in leaves, fruits and bark of the Fagaceae. According to Hegnauer (2001), also methylated ellagic acids occur. In hydrolysable tannins glucose may be replaced by hamamelose, quercitol, further polyalcohols (shikimi acid, quinic acid, proto-quercitol, scyllo-quercitol, salidroside, 3,4,5-trihydroxybenzylalcohol) or phenolics.	
Flavonoid compounds are characteristic of the Fagaceae, even often occurring in the wood. The pattern of flavonoids strongly depends on the plant part investigated. As to the leaves, the flavonoid pattern might change drastically with seasons.	
Quercus	
Tannins: Condensed tannins (polymeric and oligomeric proanthocyanidins) are	

[1] robinobiose = 6-O-(6-deoxy-α-L-mannopyranosyl)-D-galactose; 6-O-α-L-rhamnopyranosyl-D-galactose

family, genus, species and plant part	classification
abundant in barks (e.g. Quercus cortex of *Quercus robur* or *Q. petrea*). Leaves contain condensed (catechin, gallocatechin, leucodelphinidin and leucocyanidin units) and hydrolysable (gallo- and ellagi-) tannins. Except for ellagitannins (pedunculagin, vescalagin, castalagin), most polyphenolic compounds of the leaves increase with seasons (from spring to autumn). In some species complex tannins (condensation products of catechins with ellagitannins) also occur in fresh barks and leaves. Gallotannins can often occur in the galls. Ellagitannins are abundant in woods (e.g. castalagin and vescalagin) and leaves. Fruits sometimes contain valonea acid. Fruit galls may contain ellagitannins (e.g. pedunculagin).	
While quercetin is abundant in leaves, kaempferol and hydroxycinnamic acids (caffeic acid, p-coumaric acid) occur irregularly. The occurrence of 5-deoxyflavones, isoflavones and dihydrochalcones is noticeable (Hegnauer, 2001).	
Triterpenoids occur in bark, especially in the cork of *Q. suber* (cork oak), but also in leaves and the coating (resins) of leaf galls of some species. Saponins may occur in bark, wood and leaves.	
Coumarins (scopoletin) and benzoic acids (vanillin-, syringa-, gentisin-,salicylic-, protocatechuic-, and m-and p-hydroxybenzoic acid) were isolated from twigs of *Q. robur*.	
Lignans (lyonisid, lyoresinol and syringaresinol), various phenolic glycosides (e.g. galloylarbutin derivatives) and 2,6-dimethoxy-p-benzoquinone are also found in *Quercus*.	
Characteristic of the genus *Quercus* is quercitol (a cyclitol), (Frohne and Jensen, 1992; Rimpler, 1999).	
<u>drugs and medicinal use</u>: Turkish galls from *Quercus infectoria* are used as a source of Acidum tannicum. For medicinal use see *Rhus*.	
In domestic medicine, various North American oaks (e.g. *Quercus falcata* and a number of other species native to North America) are recommended for diarrhoea, swellings and skin ailments (bark, leaves, acorns), (Crellin and Philpott, 1990).	
Quercus falcata Michx.	
leaves and bark	tasted
Quercus phellos L.	untouched
Quercus stellata Wagenh.	
leaves and bark	tasted
Hamamelidaceae (order: Hamamelidales)	
Polyphenolic compounds and acids are characteristic of the family: tannins are abundant in leaves, barks and woods.	
Both, condensed and hydrolysable (gallo-) tannins occur in leaves and barks: e.g. Hamamelidis cortex (*Hamamelis virginiana*) contains hamamelitannin (= digalloylhamamelose), monogalloylhamameloses and condensed tannins (total content up to 12 %), (Hegnauer, 2001; Jänicke *et al.*, 2003).	
Glycosides of myricetin, quercetin and kaempferol are abundant in leaves (Hegnauer, 2001).	
<u>drugs</u>: Hamamelidis cortex et aqua (*H. virginiana*);	
Styrax (= Storax) liquidus (*Liquidambar orientalis*) consists of < 1% essential oils and non-volatile constituents. The oleoresin styrax is obtained from *Liquidambar formosana*;	
"American Styrax" (or sweet gum) from *Liquidambar styraciflua* contains 15-20% essential oils (Hegnauer, 2001).	
Liquidambar	
A genus that contains resins and oleoresins which are typically manufactured once the plant is injured (known as styrax or storax) (Hegnauer, 2001).	

family, genus, species and plant part	classification
Liquidambar styraciflua L.	
drugs: American Styrax	
medicinal and other use: In pioneers days, the gum was obtained from the trunks by peeling the bark and scraping off the resin-like solid. This gum was used for chewing gum (Little, 1993), and medicinally in pharmaceuticals for treating skin lesions, sores, catarrh, and dysentery (Hough´s Encyclopaedia, 1958), and in the Compound Tincture of Benzoin. An ointment containing American gum was prepared for haemorrhoids, itch, ringworm, catarrh, and other chest ailments. Leaves and bark were recommended for coughs and colds. The bark was recommended as tonic and used for upset stomach. A decoction of the inner bark was used as mucilaginous astringent for e.g. diarrhoea and dysentery (Crellin and Philpott, 1990).	
leaves	eaten
flavonoids: 1.0 mg myricetin, 1.5 mg quercetin, 0.1 mg kaempferol (referred to 1 g fresh weight). Myricitrin (myricetin-3-rhamnoside) and quercitrin (quercetin-3-rhamnoside) are abundant.	
tannins: gallo- and ellagitannins, bergenin (associated gallic acid derivative) (Hegnauer, 2001);	
anthocyanins	
essential oils: 0.08 % (dried leaves); 0.125 % (fresh leaves), e.g. α-pinene, limonene, cineol, 1-terpinen-4-ol, sabinene, vitispirane, valeranone, valerenal and presumably borneol and bornylacetat (Hegnauer, 2001; Tattje and Bos, 1979; Tattje *et al.*, 1980; Wyllie and Brophy, 1989); the oil consists of about 85 % mono- and sesquiterpene hydrocarbons and about 15 % oxygenated compounds (Tattje *et al.*, 1980);	
cyanogenic compounds: weak cyanogenesis was found in only some of the leaf samples studied (Hegnauer, 2001)	
wood	
unknown compound: presumably a lipophilic, skin-irritating compound (Hegnauer, 2001)	
leaves, barks and fruits	
organic acids: shikimi acid (Hegnauer, 2001)	
leaves, barks, wood, flowers, fruits	
heteroside (pseudoindikan): monotropein (Hegnauer, 2001)	
Juglandaceae (order: Juglandales)	
The family is characterised by polyphenols, tannins and naphthoquinones. Condensed and hydrolysable (gallo- and ellagi-) tannins occur differently in fruits, seeds, barks and leaves.	
Naphthoquinones (especially juglone and the genuine hydrojuglone-ß-glucoside, e.g. abundant in leaves and other organs of *Juglans* and *Carya* species) exhibit fungitoxic, phytotoxic and ichthyotoxic effects (Frohne and Jensen, 1992; Hegnauer, 2001). In addition, juglone shows laxative effects and causes pustules on skin surface (Hegnauer, 2001).	
Essential oils occur in leaves (e.g. of *Juglans regia*; with fungistatic effects; it contains various terpenoids, phenylpropanoid compounds, alcanes and fatty acids) (Frohne and Jensen, 1992).	
Myricetin, quercetin, kaempferol and dihydrokaempferol are abundant, in part methylated, often as 3-rhamnosid or 3-arabinosid glycosides in barks, twigs and leaves. Also bi- and triflavonoids occur. Hydroxycinnamic acids are found. Cyclitols are abundant in leaves (of genera *Juglans* and *Carya*).	
Ascorbic acid is abundant in leaves and unripe fruits of *J. regia* (Hegnauer, 2001).	
Carya tomentosa (Poir.) Nutt.	
leaves	tasted
naphthoquinones: 0.5 mg per g dry weight hydrojuglone (Hegnauer, 2001)	
bark	
flavonoids: presumably quercitrin (Hegnauer, 2001)	

family, genus, species and plant part	classification
Magnoliaceae (order: Magnoliales)	
Essential oils are characteristic of the family (monoterpenes, sesquiterpenes, phenylpropanes). Alkaloids derived from phenylalanine are abundant (phenylethylamine derivatives, benzyltetrahydroisochinolin bases, aporphin bases and oxidised derivatives, berberin bases, bisbenzylisochinolin bases and quaternary compounds). Flavonoids (quercetin and kaempferol glycosides), coumarins, hydoxycinnamic acids (caffeic acid) and lignans are abundant. Condensed tannins (leucocyanidin units) occur sometimes. Ellagitannins were not found. Cyanidin and paeonidin glycosides often occur in flowers. Various cyclitols are known to occur in leaves (Hegnauer, 2001).	
drugs: Barks of various species of the Magnoliaceae are used in China against gastrointestinal disorders (their alkaloids show in part spasmolytic activity (Hegnauer, 2001).	
Liriodenderon	
leaves, bark, root bark and / or fruits	
sesquiterpene lactones (biologically active): parthenolide, custolide and further constituents of germacranolide-, eudesmanolide-, elemanolide- and guaianolide-type (Hegnauer, 2001)	
bark	
tannins (Hegnauer, 2001)	
Liriodendron tulipifera L.	
medicinal use: Bark and root bark have been used as tonic and stomachic, for rheumatism and fever. The drug was even included in the USP (Crellin and Philpott, 1990).	
protoalkaloids and alkaloids: aporphin-type (several compounds) and their various oxidation products; proaporphin-type; benzoe acid amide-type; benzylisochinolin bases; tetrahydroprotoberberin-alkaloids (Hegnauer, 2001).	
leaves	eaten
flavonoids: quercetin and kaempferol glycosides are abundant: isoquercitrin, rutin, kaempferol-3-glucoside and -3-rutinoside (Hegnauer, 2001);	
hydroxycinnamic acids: caffeic acid; chlorogenic acid (Hegnauer, 2001);	
bisphenylpropane-derivative: 1-(3,4,5-trimethoxyphenyl)-2-(4-allyl-2,6-dimethoxy)propane (Barbosa *et al.*, 1990);	
tannins: condensed tannins (leucocyanidin units) (Hegnauer, 2001);	
cyanogenic compounds were determined as HCN in fresh leaves. Large amounts with seasonal differences were found: May: 490 mg / kg HCN; June: 225-248 mg / kg; August: 100 mg / kg. Leaves contained taxiphyllin and triglochinin (Hegnauer, 2001).	
saponin-like substances (Hegnauer, 2001);	
alkaloids (especially aporphin bases and related compounds): glaucin; liridin, liridinin (Hegnauer, 2001); N-methylcrotsparine (Barbosa *et al.*, 1990);	
essential oils / sesquiterpene lactones: of germacranolide-, eudesmanolide-, elemanolide- and guaianolide-type; tulirinol (of germacranolide-type; an antifeedant for the gypsy moth larvae) (Hegnauer, 2001; Barbosa *et al.*, 1990); lipiferolide (Barbosa *et al.*, 1990);	
cyclitols: D-ononitol, D-bornesitol (myo-inositol methyl ether, in young leaves); liriodendritol (myo-inositol-1,4-dimethyl ether, in summer leaves); 2-C-methyl-erythritol (in autumn leaves) (Hegnauer, 2001);	
compounds not found by leaf analyses: myricetin, leucodelphinidin and ellagic acid; leaves contain absolutely no silica (Hegnauer, 2001)	
leaf, bark, flower, fruits	
cyclitols: liriodendritol, which is accompanied by low amounts of the 1-monomethyl and 4-monomethylether (Hegnauer, 2001)	

family, genus, species and plant part	classification
bark hydroxycinnamic acids: syringa acid methyl ester; coumarins: aesculetin dimethylether (Hegnauer, 2001); lignans and neolignans: lioresinol diglucoside (isomer of syringaresinol) from the inner bark; liriodendron, pinoresinol, syringaresinol, lirionol; alkaloids (especially aporphin bases and related compounds): liriodenin; glaucin; N-nornuciferin, O-methyl-N-norlirinin; benzoe acid amide; cardioactive compounds: presumably sesquiterpene lactones (Hegnauer, 2001) root bark essential oils: sesquiterpene lactones (types like in leaves) (Hegnauer, 2001) wood and heartwood alkaloids (especially aporphin bases and related compounds): liriodenin (antimicrobial); dehydroglaucin, michelalbin, N-acetyl-N-nornantenin and its 3-methoxy-derivative, liriodendronin (Hegnauer, 2001) **Mimosaceae** (order: Fabales) drugs: Catechu (containing condensed tannins) (*Acacia catechu*) and barks of other *Acacia* species are used as source of tannins; Gummi arabicum, Acaciae gummi (Ph. Eur.; an arabinogalactan) is gained from *Acacia* species (e.g. *A. senegal*) as excretion of the trunk; it is used as emulsifying agent and stabiliser in the pharmaceutical technology and food chemistry; in the production of adhesives and inks (Frohne and Jensen, 1992; Rimpler, 1999). *Albizia* Gummosis and exudates (mostly acidic reacting) were found in some species of the genus *Albizia*: *A. anthelmintica*, *glaberrima*, *lebbeck*, *procera*, *sericocephala*, *zygia*. (Anderson *et al.*, 1966; Anderson and Morrison, 1990; Martínez *et al.*, 1995; review given by Hegnauer, XIa, 1994; for *A. zygia* see Mital and Adotey, 1971;1972;1973). Characterisation of the protein moiety revealed that aspartic acid and proline were main components, but also hydroxyproline may be important (Hegnauer, XIa, 1994). Barks of some *Albizia* species (e.g. *A. chinensis*, *granulosa*, *lebbeck*, *procera*) contain 5-25 % tannins (Hegnauer, XIa, 1994). Saponins are abundant in the genus *Albizia* (largely in barks, but also in other plant organs like leaves, flowers, pods, seeds, wood) exhibiting various ecological and pharmacological properties (Meyer *et al.*, 1982; Carpani *et al.*, 1989; Orsini *et al.*, 1991; Pal *et al.*, 1995; a review is given by Hegnauer, XIb-1, 1996; Yoshikawa *et al.*, 1998a). Alkaloids (budmunchiamines, that is macrocyclic spermine alkaloids) occur in some *Albizia* species; e.g. in the seeds of *A. amara* (Mar *et al.*, 1991; Pezzuto *et al.*, 1992), the seeds of *A. lebbeck* (N-demethyl-budmunchiamine alkaloids; Misra *et al.*, 1995), in the stem bark of *A. gummifera* and *A. schimperana* (Rukunga and Waterman, 1996a and b) with e.g. antibacterial and antiviral activity, cytotoxic and immunological effects (Mar *et al.*, 1991; Pezzuto *et al.*, 1992; Rukunga and Waterman, 1996a and b). medicinal use: The stem bark of *A. gummifera* is used in traditional Kenyan medicine for the treatment of coughs, gonorrhoea, fever, malaria, stomach pains and skin diseases (Watt and Breyer-Brandwijk, 1962; Kokwaro, 1976).	

family, genus, species and plant part	classification
Albizia julibrissin	
drugs:	
Albiziae cortex (Chinese Pharmacopoeia; Stöger *et al.,* 2001):	
Leading compounds are saponins with haemolytic activity.	
Albiziae flos (Chinese Pharmacopoeia; Stöger *et al.,* 2001):	
The Pharmacopoeia tests for lipophilic compounds referenced to authentic plant material.	
According to Pharmacopoeia, both drugs are used to loose stases and as sedative agent, against restlessness, agitation, sleeplessness and despondency. Moreover, the bark is used against ulceration in the "orbis pulmonalis" and externally against ulcers, swellings, fall and strike injuries.	
The saponin fraction of the stem bark is reported to show a strong uterotonic activity by stimulating uterine muscle contractions (You *et al.*, 1982; Tang and Eisenbrand, 1992). Furthermore, the crude saponin fraction exhibited significant cytotoxicity with julibroside III as most potent compound (Ikeda *et al.*, 1997).	
According to Kinjo *et al.* (1991a and b) the stem bark is used as tonic in China and Japan and it is syringaresinol diglucoside (a lignan glycoside) which is made responsible for the pharmacological effect "to ease the mind and calm the nerves". Moreover, the bark is used as antipyretic, anodyne, sedative and diuretic drug, which might be due to the N-acetyl-D-glucosamine sugar moiety of saponin glycosides (Kinjo *et al.*, 1992).	
Flowers are used as a sedative drug, for quercitrin and isoquercitrin contained sedative activity has been found (Kang *et al.*, 2000).	
remark	
This tree is often called "Mimosa-tree" because the flowers are similar to those of the related herbaceous sensitive-plants (genus *Mimosa*). Silktree (*A. julibrissin*) leaflets fold up at night; those of sensitive-plants fold up when touched (Little, 1993).	
plant part not specified	
flavonoids: quercetin-3-O-galactoside (hyperoside); quercetin-3-O-rhamnoside (quercitrin), (Kaneta *et al.,* 1980; Hegnauer, XIa, 1994);	
polysaccharides with anti-tumour activity (Moon *et al.*, 1985);	
enzymes: milk-clotting (protease-like) enzymes (Ohtani, 1989);	
phenanthrene derivative: tylophorine B (= 2,3,6-trimethoxyphenanthro [9,10-f]indolizidine (Wang *et al.*, 2000)	
leaves	eaten (heavily eaten in spring, less eaten in autumn)
flavonoids: quercitrin and afzelin (which both showed free radical scavenging activity), (Jang *et al.*, 2002);	
hydroxycinnamic acids: 3-O-(4-hydroxy-trans-cinnamoyl) quinic acid (= p-cumaroyl quinic acid);	
3-O-(3,4-dihydroxy-trans-cinnamoyl)-4-O-malonyl-quinic acid (= 3-caffeoyl-4-malonyl quinic acid) (Schaller and Schildknecht, 1992)	
flower	
essential oils: linalool; cis- and trans-linalool oxide; α-ocimene; isopentanol; 4-penten-2-one; 2,2,4-trimethyloxetane (Chao *et al.*, 1988);	
anthocyanins: cyanidin-3-O-ß-D-glucosopyranoside (Ishikura *et al.*, 1978; Tang and Eisenbrand, 1992);	
flavonoids: quercitrin and isoquercitrin (Kang *et al.*, 2000)	
stem bark	
flavonoids: 3',4',7-trihydroxyflavone (Chamsuksai *et al.*, 1981);	
5-deoxyflavone (geraldone), isookanin, luteolin, an isoflavone (daidzein), prenylated flavonoids (sophoflavescenol, kurarinone, kurarinol, kuraridin, kuraridinol) (Jung *et al.*, 2004a);	

family, genus, species and plant part	classification
sapogenols (triterpenoid): machaerinic acid methylester; acacic acid lactone (You *et al.*, 1982); acacigenin B; machaerinic acid lactone (Kang and Woo, 1983); 16-deoxyacacigenin B (= 21-[4-(ethylidene)-2-tetrahydrofuranmethacryloyl]machaerinic acid), (Woo and Kang, 1984; Tang and Eisenbrand, 1992); julibrogenin B and julibrogenin C, (Kinjo *et al.*, 1992); saponins (triterpenoid): julibroside A_1: acacic acid lactone 3-O-ß-D-xylopyranosyl-(1→2)-ß-D-fucopyranosyl-(1→6) [ß-D-glucopyranosyl-(1→2)]-ß-D-glucopyranoside, (Kinjo *et al.*, 1992); julibroside A_2: acacic acid lactone 3-O-ß-D-xylopyranosyl-(1→2)-ß-D-fucopyranosyl-(1→6)-ß-D-glucopyranoside, (Kinjo *et al.*, 1992); julibroside A_3: acacic acid lactone 3-O-ß-D-xylopyranosyl-(1→2)-ß-D-fucopyranosyl-(1→6)-2-acetylamino-2-deoxy-ß-D-glucopyranoside, (Kinjo *et al.*, 1992); julibroside A_4: 3-O-ß-D-glucopyranosyl-(1→4)-ß-D-glucopyranosyl-acacic acid lactone 16-O-ß-D-glucopyranoside, (Kinjo *et al.*, 1992); julibroside C_1: julibrogenin C 3-O-[ß-D-xylopyranosyl-(1→2)-ß-D-fucopyranosyl-(1→6)] [ß-D-glucopyranosyl-(1→2)]-ß-D-glucopyranoside, (Kinjo *et al.*, 1992); (julibroside A_1-A_4, B_1 and C_1: 2-15 ppm, respectively; Hegnauer, XIb-1, 1996) julibroside I: 21-O-[(6S)-2-trans-2,6-dimethyl-6-O-(4-O-(6S')-2'-trans-2',6'-dimethyl-6'-O-ß-D-quinovopyranosyl-2',7'-octadienoyl-ß-D-quinovopyranosyl)-2,7-octadienoyl]-3-O-{ß-D-glucopyranosyl-(1→2)-[ß-D-xylopyranosyl-(1→2)-ß-D-fucopyranosyl-(1→6)]-ß-D-glucopyranosyl} acacic acid 28-O-α-L-arabinofuranosyl-(1→4)-[ß-D-glucopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester; showed anti-tumour activity, (Ikeda *et al.*, 1995b); julibroside II: 21-O-[(6S)-2-trans-2,6-dimethyl-6-O-(4-O-(6S')-2'-trans-2',6'-dimethyl-6'-O-ß-D-quinovopyranosyl-2',7'-octadienoyl-ß-D-quinovopyranosyl)-2,7-octadienoyl]-3-O-[ß-D-xylopyranosyl-(1→2)-ß-D-fucopyranosyl-(1→6)-ß-D-glucopyranosyl] acacic acid 28-O-α-L- arabinofuranosyl-(1→4)-[ß-D-glucopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester, (Ikeda *et al.*, 1995a); julibroside III: 21-O-[(6S)-2-trans-2,6-dimethyl-6-O-(4-O-(6S')-2'-trans-2',6'-dimethyl-6'-O-ß-D-quinovopyranosyl-2',7'-octadienoyl-ß-D-quinovopyranosyl)-2,7-octadienoyl]-3-O-[ß-D-xylopyranosyl-(1→2)-ß-D-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-ß-D-glucopyranosyl] acacic acid 28-O-α-L-arabinofuranosyl-(1→4)-[ß-D-glucopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester, (Ikeda *et al.*, 1995a); showing pronounced cytotoxic activity (Ikeda *et al.*, 1997); julibroside J_1: 3-O-[ß-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-ß-D-glucopyranosyl]-21-O-{(6S)-2-trans-2-hydroxymethyl-6-methyl-6-O-[4-O-((6S)-2-trans-2,6-dimethyl-6-O-(6-deoxy-ß-D-glucopyranosyl)-2,7-octadienoyl)-6-deoxy-ß-D-glucopyranosyl]-2,7-octadienoyl}-acacic acid 28-O-{ß-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)}-ß-D-glucopyranosyl ester, (Ma *et al.*, 1996). The strucure of julibroside J_1 was revised by Zou *et al.* (2000a) as (6R)-configuration in the outer monoterpene moiety:	

family, genus, species and plant part	classification
3-O-[ß-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-ß-D-glucopyranosyl]-21-O-{(6S)-2-trans-2-hydroxymethyl-6-methyl-6-O-[4-O-((6R)-2-trans-2,6-dimethyl-6-O-(ß-D-quinovopyranosyl)-2,7-octadienoyl)-ß-D-quinovopyranosyl]-2,7-octadienoyl}-acacic acid-28-O-ß-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester. julibroside J_9 was established as the (6S/6S)-diastereoisomer of julibroside J_1. Both saponins exhibited cytotoxic activity (Zou *et al.*, 2000a); julibroside J_5: 3-O-[ß-D-xylopyranosyl-(1→2)-ß-D-fucopyranosyl-(1→6)-ß-D-glucopyranosyl]-21-O-{(6S)-2-trans-2-hydroxymethyl-6-methyl-6-O-[4-O-((6R)-2-trans-2,6-dimethyl-6-O-(ß-D-quinovopyranosyl)-2,7-octadienoyl)-ß-D-quinovopyranosyl]-2,7-octadienoyl}-acacic acid-28-O-ß-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester, (Zou *et al.*, 2005a); julibroside J_6: 3-O-[ß-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-ß-D-2-deoxy-2-acetamidoglucopyranosyl]-21-O-{6S-2-trans-2-hydroxymethyl-6-methyl-6-O-[4-O-(6S-2-trans-2-hydroxymethyl-6-methyl-6-hydroxy-2,7-octadienoyl)-ß-D-quinovopyranosyl]-2,7-octadienoyl}-acacic acid-28-O-ß-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester, (Zou *et al.*, 2000b); julibroside J_8 (Zou *et al.*, 2005a): (6S/6S)-diastereoisomer of julibroside J_5. Julibroside J_8 showed marked cytotoxic activity; julibroside J_{10} and J_{11} (Zou *et al.*, 2004a): a pair of diastereoisomers; julibroside J_{12}: 3-O-[ß-D-xylopyranosyl-(1→2)-ß-D-fucopyranosyl-(1→6)-ß-D-2-deoxy-2-acetamidoglucopyranosyl]-21-O-{(6S)-2-trans-2-hydroxymethyl-6-methyl-6-O-[4-O-((6R)-2-trans-2,6-dimethyl-6-O-(ß-D-quinovopyranosyl)-2,7-octadienoyl)-ß-D-quinovopyranosyl]-2,7-octadienoyl}-acacic acid-28-O-ß-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester, (Zou *et al.*, 2005a); julibroside J_{13} (Zou *et al.*, 2005a): (6S/6S)-diastereoisomer of julibroside J_{12}; Julibroside J_{13} showed marked cytotoxic activity. julibroside J_{22}: 3-O-[ß-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-ß-D-2-deoxy-2-acetamidoglucopyranosyl]-21-O-[(6S)-2-trans-2-hydroxymethyl-6-methyl-6-O-ß-D-quinovopyranosyl-2,7-octadienoyl]-acacic acid-28-O-ß-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester; showed marked cytotoxic activity, (Zou *et al.*, 2005b); julibroside J_{23} (Zou *et al.*, 2000c); julibroside J_{24}: 3-O-ß-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-ß-D-glucopyranosyl-21-O-{(6S)-2-trans-2-hydroxymethyl-6-methyl-6-O-[3-O-(6S)-2-trans-2-hydroxymethyl-6-methyl-6-hydroxy-2,7-octadienoyl-ß-D-xylopyranosyl]-2,7-octadienoyl}-acacic acid-28-O-ß-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester, (Zou *et al.*, 2004b); julibroside J_{25}: 3-O-[ß-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-ß-D-glucopyranosyl]-21-O-[(6S)-2-trans-2-hydroxymethyl-6-methyl-6-O-ß-D-quinovopyranosyl-2,7-octadienoyl]-acacic acid-28-O-ß-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-ß-D-	

family, genus, species and plant part	classification
glucopyranosyl ester; showed marked cytotoxic activity (Zou *et al.*, 2005b);	
julibroside J_{26}: 3-O-[ß-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-ß-D-glucopyranosyl]-21-O-[(6S)-2-trans-2-hydroxymethyl-6-methyl-6-O-ß-D-quinovopyranosyl-2,7-octadienoyl]-16-deoxy-acacic acid 28-O-ß-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester, (Zou *et al.*, 1999);	
julibroside J_{27}: 3-O-ß-D-xylopyranosyl-(1→2)-ß-D-fucopyranosyl-(1→6)-ß-D-glucopyranosyl-21-O-{(6S)-2-trans-2-hydroxymethyl-6-methyl-6-O-[4-O-((6S)-2-trans-2-hydroxymethyl-6-methyl-6-hydroxy)-2,7-octadienoyl-ß-D-quinovopyranosyl]-2,7-octadienoyl}-acacic acid-28-O-ß-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester; showed cytotoxic activity, (Zou *et al.*, 2000d);	
3-O-[ß-D-xylopyranosyl-(1→2)-ß-D-fucopyranosyl-(1→6)-ß-D-glucopyranosyl]-21-O-6(S)-2-trans-2-hydroxymethyl-6-methyl-6-O-ß-D-quinovopyranosyl-2,7-octadienoyl-acacic acid-28-O-L-arabinofuranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-ß-D-glucopyranosyl ester, (Zheng *et al.*, 2004);	
<u>lignan glycosides and related compounds</u>:	
(according to Hegnauer (XIb-1, 1996) yielding 20-140 ppm, respectively)	
(-)-syringaresinol-4-O-ß-D-glucopyranoside;	
(-)-syringaresinol-4,4'-bis-O-ß-D-glucopyranoside (= syringaresinol diglucoside = liriodendrin);	
(-)-syringaresinol-4-O-ß-D-apiofuranosyl-(1→2)-ß-D-glucopyranoside;	
(-)-syringaresinol-4-O-ß-D-apiofuranosyl-(1→2)-ß-D-glucopyranosyl-4'-O-ß-D-glucopyranoside, (Kinjo *et al.*, 1991a);	
(-)-syringaresinol-4,4'-bis-O-ß-D-apiofuranosyl-(1→2)-ß-D-glucopyranoside, (Kinjo *et al.*, 1991a);	
syringic acid methyl ester 4-O-ß-D-apiofuranosyl-(1→2)-ß-D-glucopyranoside;	
glaberide I 4-O-ß-D-glucopyranoside;	
glaberide I 4-O-ß-D-apiofuranosyl-(1→2)-ß-D-glucopyranoside, (Kinjo *et al.*, 1991b);	
(+)-5,5'-dimethoxylariciresinol 4-O-ß-D-apiofuranosyl-(1→2)-ß-D-glucopyranoside (Kinjo *et al.*, 1991b);	
5,5'-dimethoxy-7-oxolariciresinol 4'-O-ß-D- apiofuranosyl-(1→2)-ß-D-glucopyranoside (the latter 5 compounds were considered as key intermediates in the biodegradation pathway of syringaresinol, (Kinjo *et al.*, 1991b);	
5,5'-dimethoxy-7-oxolariciresinol (Tong *et al.*, 2003);	
(+)-lyoniresinol 9'-O-ß-D-glucopyranosyl-(1→4)-ß-D-glucopyranoside;	
(+)-lyoniresinol 4,9'-bis-O-ß-D-glucopyranoside;	
icariside-E_5 (a neolignan glucoside) (Higuchi *et al.*, 1992a; Hegnauer, XIb-1, 1996);	
<u>phenolic glycosides</u>:	
3,4,5-trimethoxyphenol 1-O-ß-D-apiofuranosyl-(1→2)-ß-D-glucopyranoside (a phloroglucin derivative, triketide) (Higuchi *et al.*, 1992a);	
albibrissinoside A and B (Jung *et al.*, 2004b);	
<u>ionon-derivative</u>:	
vomifomiol 3'-O-ß-D-apiofuranosyl-(1→6)-ß-D-glucopyranoside (Higuchi *et al.*, 1992a);	
<u>pyridoxine derivatives</u>:	
3-hydroxy-5-hydroxymethyl-4-methoxymethyl-2-methylpyridine 3-O-ß-D-glucopyranoside (a neurotoxin) (Higuchi *et al.*, 1992b; Hegnauer, XIb-1, 1996);	
julibrine I: 3-hydroxy-5-hydroxymethyl-4-methoxymethyl-2-methylpyridine 5'-	

family, genus, species and plant part	classification
O-ß-D-glucuronopyranosyl(1→2)ß-D-glucopyranoside (Higuchi *et al.*, 1992b); julibrine II (arrhythmic inducing agent): 3-hydroxy-5-hydroxymethyl-4-methoxymethyl-2-methylpyridine 5'-O-ß-D-apiofuranosyl (1→2) ß-D-glucopyranoside (Higuchi *et al.*, 1992b); <u>sterols</u>: α-spinasteryl-D-glucoside; Δ^7-stigmastenol (Chamsuksai *et al.*, 1981); <u>cerebroside</u>: soya-cerebroside I (Jung *et al.*, 2004a); <u>acids</u>: (E)-4-hydroxy-dodec-2-enedioic acid (unsaturated hydroxy acid; Jung *et al.*, 2003); (6R)- and 6S-trans-2,6-dimethyl-6-O-ß-D-quinovosyl-2,7-menthiafolic acid[2] (Tong *et al.*, 2003) root bark <u>flavonoids</u>: luteolin-7-O-neohesperidose (Go *et al.*, 2004); <u>lignan glycosides and related compounds</u>: (+)-medioresinol (moderate cytotoxic activity); (-)-syringaresinol (Go *et al.*, 2004); <u>others</u>: euscaphic acid ester glucoside, (Go *et al.*, 2004) pods <u>essential oil</u> (Lu *et al.*, 2003) seeds <u>flavonoids</u>: 3,5,4'-trihydroxy, 7,3'-dimethoxy-3-O-ß-D-glucopyranosyl-α-L-xylopyranoside (Yadava and Reddy, 2001); <u>enzymes</u>: proteinase inhibitors (Odani *et al.*, 1979; Odani *et al.*, 1980; Ohtani, 1989); <u>non-protein amino acids</u>: S-(ß-carboxyethyl)-cystein, albizziin (Hegnauer, XIb-1, 1996)	
Moraceae (order: Urticales) Polyphenolic compounds are characteristic of leaves, barks, woods and fruits with in part pharmacological effects on various organisms (Hegnauer, 2001): hydroxycinnamic acids, flavonoids, benzophenones, xanthones, stilbenes and coumarins. (Insecticidal and fungicidal stilbenes and xanthones occur in some woods. Some phenolic compounds act as phytoalexines.) Condensed tannins are restricted to some genera, gallo- and ellagitannins are nearly absent. Quercetin and kaempferol glycosides are abundant in leaves. Some rare and typical flavonoid compounds occur (Hegnauer, 2001). Latex is a characteristic: which contains finely dispersed polyisoprenoids and is rich in proteins and proteinases. Other compounds of the latex (like cardenolid glycosides) vary considerably between genera and species. Cardenolid glycosides may also occur in seeds, barks and woods of individual species. Caoutchouc is gained from some species (Frohne and Jensen, 1992; Hegnauer, 2001). Mineralisation is characteristic of the entire order Urticales (including Moraceae and Ulmaceae): calcium oxalate and silicates are abundant in leaves (Frohne and Jensen, 1992). Acidic plant mucilage is abundant in the order (Frohne and Jensen, 1992). Triterpenoids, sterols and waxes occur in part in latices, in part in adjacent plant tissue (Hegnauer, 2001). Alkaloids may occur (as flavonoidalkaloids in *Ficus*) (Hegnauer, 2001). <u>usage</u>: Latices leading cardenolid-glycosides are used as arrow poison by native people.	

[2] (6R/S)-menthiafolic acid: (6R/S)-2-trans-2,6-dimethyl-6-hydroxy-2,7-octadienoic acid; monoterpene moiety e.g. in julibrosides

family, genus, species and plant part	classification
Morus	
drugs: Mori cortex radicis (*Morus alba*), remedy in China and Japan (prenylated and geranylated (bi)flavonoids show hypotensive effects) (Hegnauer, 2001).	
leaves	
plant acids: fumaric, succinic, malonic, malic, citric, oxalic acid;	
flavonoids: moracetin (quercetin-3-triglucoside);	
hydroxycinnamic acids: chlorogenic acid, p-coumaric acid;	
sterols: ß-sitosterol and compounds esterified with fatty acids; inokosteron and ecdysteron (Hegnauer, 2001);	
polyprenones: bombiprenone (Hegnauer, 2001)	
bark	
triterpenoids: amyrin, amyrinacetat, betulinic acid (Hegnauer, 2001);	
sterols: sitosterol (Hegnauer, 2001);	
prenylated flavonoids, benzofuran-derivatives and coumarins (see root bark) (Hegnauer, 2001);	
alkaloids: piperidinalkaloid (Hegnauer, 2001)	
root bark	
flavonoids: prenylated and geranylated flavonoids and benzofuran derivatives (various chalcone-flavone-, chalcone-flavanon-, chalcone-phenylbenzofuran-, flavone-flavone-adducts with in part hypotensive effects) (Hegnauer, 2001);	
coumarins: prenylated and methylated compounds;	
xanthones: prenylated and methylated compounds (Hegnauer, 2001);	
alkaloids: piperidinalkaloids: nojirimycin exhibiting antibiotic effects; moranolin (Hegnauer, 2001)	
wood (of trunks and / or twigs)	
flavonoids: morin, dihydromorin, dihydrokaempferol (aglyca);	
benzophenones and xanthones: maclurin, 2,4,4´,6-tetrahydroxybenzophenone;	
stilbenes: 2,4,3´,5´-tetrahydroxystilbene;	
polyphenols: albolactol (Hegnauer, 2001)	
Morus rubra	
medicinal use: In folk medicine laxative and anthelminthic properties are reported for the fruit and especially the bark (Crellin and Philpott, 1990).	
leaves	eaten
flavonoids: flavonol glycosides, dihydroquercetin (aglycone) (Hegnauer, 2001);	
latex (Hegnauer, 2001)	
bark	
flavonoid-benzofuran-adduct: albanol-A = mulberrofuran G (Hegnauer, 2001);	
flavonoids: rubraflavones A,B,C,D (flavones with C_{10} side chains). The flavone itself possesses coronary dilating action. Derivatives have been tested as coronary vasodilatator. Others have been reported to have antiphlogistic, choleretic, spasmolytic and antihistamine activity (Venkataraman, 1975).	
wood	
simple phenolic compounds: resorcin, ß-resorcylaldehyd (Hegnauer, 2001);	
flavonoids: morin, quercetin (aglyca) (Hegnauer, 2001);	
stilbenes: oxyresveratrol (Hegnauer, 2001)	
Nyssaceae (order: Cornales)	
Iridoid (iridoid-glycosides) and secoiridoid compounds are characteristic of the family. Galli- and ellagitannins are abundant (Hegnauer, 2001). Hegnauer (2001) stresses the strong relationship to the Cornaceae.	
usage: In former times the light and porous wood " Tupelo lignum " of *Nyssa*-species was used as surgical instrument (Hegnauer, 2001).	
Nyssa sylvatica Marsh.	untouched
leaves	
flavonoids: 0.16 % myricitrin in autumn leaves; quercitrin, guajaverin, hyperoside and rutin (Hegnauer, 2001);	
tannins: ellagitannins (Hegnauer, 2001);	
triterpenoids: 0.8 % free ursolic acid (Hegnauer, 2001)	

family, genus, species and plant part	classification
leaves and twigs (stems):	
<u>iridoid compounds</u>: presumably cornin (according to Hegnauer depending on the study performed) (Hegnauer, 2001)	
Oleaceae (order: Oleales)	
Iridoid and secoiridoid compounds, iridoid glycosides and iridoid pseudoalkaloids are characteristic. The bitter tasting secoiridoid oleuropaein in the leaves of *Olea europaea* is expected to exert the anti-hypertensive effects of this drug (Frohne and Jensen, 1992). Bitter tasting compounds of the oleuropaein- and swertiamarin-type are abundant in Oleaceae (Hegnauer, 2001).	
Main phenolic compounds are ester-glycosides of the verbascosid-type with in part tannin-like effects (verbascosid itself consists of a hydroxycinnamic (caffeic) acid and a 3,4-dihydroxyphenylethanol moiety which are bound to oligosaccharides) (Frohne and Jensen, 1992), while typical tannins (condensed and / or hydrolysable) are absent (Hegnauer, 2001). According to Hegnauer, p-coumaric acid, caffeic acid, ferula acid and isoferula acid build the ester moieties, while 4-hydroxy- and 3,4-dihydroxy-phenylethanol and hydroxycinnamic alcohol build the ether moieties which are bound to mono- and oligosaccharides of these tannin-like compounds, which in turn are abundant in fruits, leaves and flowers. Mere arylalcanol- and arylalcenol-glycosides are also characteristic of the family.	
Mannitol is accumulated by most Oleaceae species in many organs (e.g. “manna” of *Fraxinus ornus*) (Frohne and Jensen, 1992; Hegnauer, 2001). Free triterpenoid acids (oleanolic- and ursolic acid) which often occur along with other triterpenoid compounds (alcohols) in leaves and fruits (mainly in cuticle waxes), flowers and seeds, and phenolic glycosides characterise the family (Hegnauer, 2001).	
Sterols are abundant in leaves, seeds and roots.	
Syringin (glycoside of a hydroxycinnamic alcohol derivative) is stored in barks and leaves of many genera and species (e.g. *Fraxinus* and *Ligustrum*). In the bark of *Fraxinus* species syringin and coumarins occur to the exclusion of one another.	
Coumarins occur in barks, as minor compounds also in leaves, woods, flowers and / or fruits of many species (Hegnauer, 2001).	
Furofuranoid lignans (e.g. phillygenin) and lignan-glucosides (e.g. phillyrin) occur in Oleaceae species (e.g. in leaves, bark, roots and / or twigs).	
Quercetin and kaempferol glycosides (especially rutin) are abundant in leaves.	
Essential oils occur in flowers of many species (Hegnauer, 2001).	
<u>drugs</u>: Fraxini folium (*Fraxinus excelsior*) serve as laxative, while Oleae folium (*Olea europaea*) is used as hypotensive agent (Hegnauer, 2001).	
Fraxinus	
Coumarins, flavonols, flavones, secoiridoid glycosides and lignans are abundant in *Fraxinus*. Generally, the coumarins aesculin and fraxin occur predominantly in barks, while only trace amounts appear in leaves (Hegnauer, 2001).	
Fraxinus americana L.	
leaves	tasted
<u>flavonoids</u>: apigenin-7-rutinoside (Hegnauer, 2001)	
seeds	
<u>secoiridoid glycosides</u>: Fraxinus-GI-3 (Hegnauer, 2001)	
Fraxinus pennsylvanica Marsh.	untouched
Ligustrum	
Secoiridoid glycosides, flavonoids, lignans and triterpenoids are characteristic of *Ligustrum* (Hegnauer, 2001).	
Ligustrum vulgare	
leaves	refused
<u>iridoid chinolin-alkaloids</u>: cinchonin, cinchonidin and dihydrocinchonidin, which are bound to verbascoside-like compounds (Hegnauer, 2001)	
fruits	
<u>secoiridoid glycosides</u>: nüzhenid, Fraxinus-GI-3 and ligustrosid are the main compounds, which is accompanied by some oleuropaein (Hegnauer, 2001);	
<u>iridoid and secoiridoid pseudoalkaloids</u>: presumably all artefacts during extraction procedure (Hegnauer, 2001)	

family, genus, species and plant part	classification
Rosaceae (order: Rosales)	
This family is known for the condensed type of tannins (leucocyanidin and catechin units). In part, also gallo- and ellagitannins occur (especially in the subfamily of Rosoideae). Ellagitannins are obviously absent in the subfamilies Maloideae and Prunoideae. Quercetin and kaempferol glycosides are abundant (Frohne and Jensen, 1992). In addition, flavones, isoflavones, coumarins, hydroxycinnamic acids and simple phenolic glycosides (e.g. arbutin) occur (Hegnauer, 2001).	
Pentacyclic triterpenes and saponins occur in cuticle waxes (leaves and fruits), triterpene alcohols and triterpene ketones in barks (Frohne and Jensen, 1992). Tormentol (= tormentill acid-28-ester glucoside) may be characteristic of the Rosaceae, which builds water-soluble complexes with tannins exhibiting antibiotic effects (Hegnauer, 2001).	
Sitosterol is abundant in the Rosaceae.	
Organic acids are abundant in fruits (predominantly malic acid and citric acid; but also succinic acid, ascorbic acid), (Frohne and Jensen, 1992).	
Cyanogenesis occurs in some subfamilies, e.g. Maloideae (*Photinia*), Prunoideae (*Prunus*): amygdalin is predominantly in seeds and / or prunasin in vegetative plant parts (Hegnauer, 2001; Frohne and Jensen, 1992).	
Epidermal cells containing mucilage often occur in the Maloideae (Hegnauer, 2001).	
drugs (selection): Due to the occurrence of tannins following drugs are used: Agrimoniae herba (*Agrimonia eupatoria*); Alchemillae herba (*Alchemilla vulgaris*); Anserinae herba (*Potentilla anserina*); Tormentillae rhizoma (*Potentilla erecta*) (Frohne and Jensen, 1992).	
Based on the occurrence of flavonoids and proanthocyanidins, different *Crataegus* species are used (Crataegi folium cum flore) against cardiac diseases (Frohne and Jensen, 1992).	
The bark of *Prunus serotina* has been officinal in the United States and England (Hegnauer, 2001).	
Photinia	
Leaves and twigs contain prunasin as cyanogenic compound. In leaves of *Photinia* species ellagitannins and condensed tannins occur. Condensed tannins (catechin units) are common among the Maloideae (Hegnauer, 2001).	
Photinia glabra Maxim.	
leaves	eaten
pentacyclic triterpenes: epifriedelanol;	
cyanogenic compounds: HCN and benzaldehyde after distillation (Hegnauer, 2001)	
Prunus	
Cyanogenic compounds: prunasin (ß-glucoside of mandelonitrile) often in leaves, barks, flowers, fruits; amygdalin in seeds. Investigated *Prunus* species yielded 0.1-1.95 % mandelic acid after hydrolysis of dried leaves and twigs.	
Flavones, isoflavones, flavanones and coumarins occur in this genus. Quercetin and kaempferol glycosides are abundant in *Prunus*: e.g. rutin, kaempferitrin, trifolin, quercitrin.	
Condensed tannins (leucocyanidin and catechin units), chalcones, flavanones, flavanonoles, flavones, isoflavones and flavonols were found in woods and barks. Coumarins occur in leaves, woods, barks and fruits (Hegnauer, 2001).	
Pentacyclic triterpenes: friedelin and epifriedelanol in barks.	
Gummosis obviously occurs in all *Prunus* species (trunk and fruit flesh) (Hegnauer, 2001).	
Prunus serotina Ehrh.	untouched
medicinal and other use: The bark of *Prunus serotina* was used as officinal drug (USP) in the United States and England. It is mentioned as remedy for hepatitis, cirrhosis, fever and circulatory disorders (Hegnauer, 2001; Crellin and Philpott, 1990). Black cherry syrup is obtained from the bark and used as cough medicine. Jelly and wine are prepared from the fruit (Little, 1993).	
The cyanogenic compounds and their products may act as allelopathic agents towards plants growing in their neighbourhood (Hegnauer, 2001).	

family, genus, species and plant part	classification
leaves flavonoids: quercetin glycosides; simple phenolics: benzoic acid, benzoylglucose; cyanogenic compounds: prunasin; 95 % benzaldehyde from fresh leaves (product originating from prunasin); paraffines, wax-alcohols; triterpenoids: ursolic acid, ursolaldehyde, 2α-hydroxy-3-epiursolic acid; essential oils: 0.4 % (from fresh leaves); sterols (Hegnauer, 2001)	
bark tannins: condensed tannins (leucocyanidin units); cyanogenic compounds: prunasin; coumarins: scopoletin; simple phenolics: benzoic acid; trimethyl gallic acid; hydroxycinnamic acids: p-coumaric acid; sterols (Hegnauer, 2001)	
wood tannins / flavanones: catechin, naringenin, 3-hydroxynaringenin (Hegnauer, 2001)	
Simaroubaceae (order: Rutales) In Rutales essential oils, bitter tasting compounds (= quassinoids, especially in Simaroubaceae) and alkaloids (canthin-6-one-alkaloids, 2-quinolone-alkaloids and ß-carbolin-type (= pyrido [3,4-b] indol-type) alkaloids) occur. Resins, plant mucilages, gums and mixtures of them are common. Quassinoids in Simaroubaceae: Quassinoids predominantly consist of a C-20 skeleton (decanortriterpenoids), but also C-18, C-19 and C-25 skeletons occur: C_{25}-quassinoids: pentanortriterpenoids, type of simarolid and of soulameolid; C_{20}-quassinoids: decanortriterpenoids, type of quassiin, the largest group of quassinoids C_{19}-quassinoids: undecanortriterpenoids, type of eurycomalactone and shinjulactone-B-type C_{18}-quassinoids: dodecanortriterpenoids, type of samaderine, (Frohne and Jensen, 1992; Hegnauer, 2001) officinal drugs: Quassiae lignum, Simaroubae cortex, Bruccae cortex et fructus pharmacological effects / medicinal use: Quassinoids show insecticidal, cytotoxic and other biological effects; bitter tasting woods, barks and seeds of Simaroubaceae are often used as bitter tonic (tonicum amarum) / stomachicum, febrifuge, antiparasitic, anthelminthic, against diarrhoea or against malaria (Crellin and Philpott, 1990; Frohne and Jensen, 1992; Hegnauer, 2001).	
Ailanthus sterols and triterpenoids: tricarbocyclic triterpenes (Hegnauer, 2001)	
Ailanthus altissima (Mill.) Swingle quassinoids (bitter tasting compounds): shinjulactone-B (Hegnauer, 2001) medicinal use: Bark and root bark are used as tonic for nerves; antidiarrhoeal and anthelminthic effects. Quassinoid constituents revealed antiprotozoal activity with possibly antimalarial effects (Crellin and Philpott, 1990).	passed
remark Male flowers have an objectionable odour; some people are allergic to their pollen (symptoms of hayfever). Roots are classed as poisonous (Little, 1993).	
leaves flavonoids: quercetin, kaempferol and apigenin as aglyca; 0.2 % isoquercitrin; hyperoside, apigenin-7-glucoside, luteolin-7-glucoside; hydroxycinnamic acids: p-coumaric acid, caffeic acid; tannins: 11.9 % tannins, gallo- and ellagitannins; alkaloids: ß-carbolin-type and canthinone-alkaloids; quassinoids (Hegnauer, 2001)	

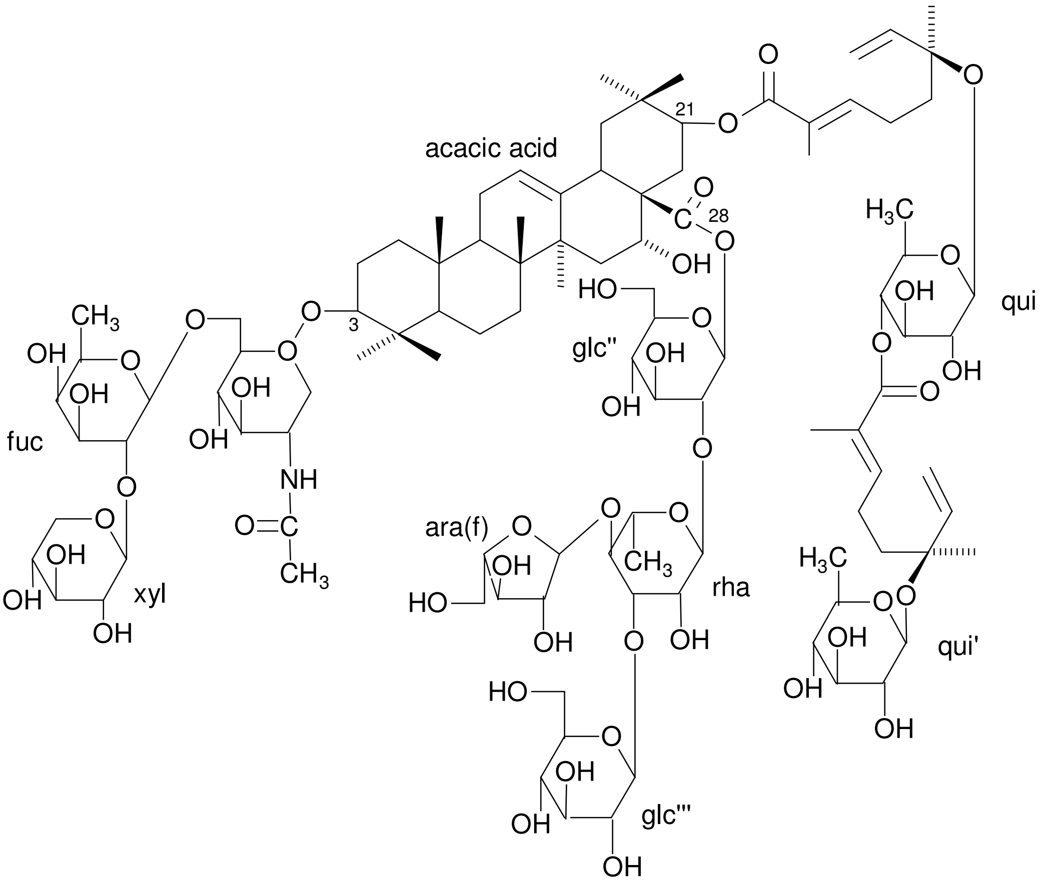
family, genus, species and plant part	classification
bark	
quassinoids (C-20 skeletons): quassiin, neoquassiin, ailanthone, amarolide, acetylamarolide;	
quinones: 2,6-dimethoxy-p-benzoquinone (bacteriostat) (Hegnauer, 2001)	
roots	
quassinoids: ailanthone;	
alkaloids: 0.05 % 1-methyl-3-dimethylallyl-4-methoxyquinolone-2 (Hegnauer, 2001)	
root bark	
quassinoids: shinjulactone-C (not bitter in taste); shinjulactone-A and -L;	
alkaloids: ß-carboline-type and canthinone-alkaloids;	
coumarins: scopoletin (Hegnauer, 2001)	
wood	
alkaloids: canthinone-alkaloids (Hegnauer, 2001)	
seeds	
quassinoids (C-20 skeletons): chaparrinone, ailanthone, ailantholide;	
quinones: 2,6-dimethoxy-p-benzoquinone (Hegnauer, 2001)	
Ulmaceae (order: Urticales)	
Gallo- and ellagitannins are nearly absent (like in Moraceae). Low contents of condensed tannins in leaves, barks and fruits. Barks, flower buds and fruits of some species are used as astringent.	
General tendency of mineralisation in Urticales, but in Ulmaceae less silicates and more calcium oxalates occur.	
Flavonoids: quercetin and kaempferol glycosides abundant in leaves; flavonoid C-glycosides in leaves and barks show in part pharmacological effects on various organisms. Hydroxycinnamic acids occur in leaves.	
Acidic plant mucilage occurs in the leaves and barks.	
Various lignans, 2,6-dimethoxybenzoquinone, different phenolic compounds including aromatic sesquiterpenes were found in woods of different species (Hegnauer, 2001).	
Ulmus	
medicinal use: Ulmi cortex (from *U. carpinifolia*, *U. glabra* and *U. rubra*) is used as mucilaginosum and antisepticum in Europe and USA (Frohne and Jensen, 1992; Hegnauer, 2001).	
leaves	
flavonoids: rutin; quercetin-3-glycosides abundant in *Ulmus*; myricetin and kaempferol also occur as aglyca (Hegnauer, 2001);	
tannins: condensed tannins (procyanidin, prodelphinidin units) (Hegnauer, 2001)	
bark	
tannins: condensed tannins; (+)-catechin-5-ß-xylosid (Hegnauer, 2001);	
triterpenoids	
wood	
various lignans and phenolic compounds (Hegnauer, 2001);	
sesquiterpenoids: aromatic sesquiterpene-alcohols (7-hydroxycalamenen; cadalin-derivatives) and quinoid compounds (mansonones); in part these sesquiterpenes function as phytoalexins with fungistatic effects (Hegnauer, 2001);	
sterols: ß-sitosterol, esterified compounds, cycloartenol and derivatives (Hegnauer, 2001)	
Ulmus alata Michx.	
leaves	refused
flavonoids: myricetin-glycosides (e.g. myricitrin) (Hegnauer, 2001)	
Vitaceae (order: Rhamnales)	
Condensed and hydrolysable tannins (ellagitannins) characterise the family (Frohne and Jensen, 1992). Phenolic acids (p-coumaric-, ferula- and caffeic acid) and aldehydes are abundant in *Vitis*. Hydroxycinnamic acids are often esterified with quinic acid, saccharides or the sugar moiety of anthocyanins. Organic acids (tartaric, oxalic, malic, acetic acid) are abundant in leaves, stalks and fruits.	

family, genus, species and plant part	classification
Vitaceae are rich in ascorbic acid (in leaves, stalks and fruits). Free pentacyclic triterpenoid acids (oleanolic acid) occur in cuticle waxes especially of fruits (grapes of *Vitis vinifera*) and only to a lesser extent in the cuticle of leaves and stalks (Hegnauer, 2001).	
Vitis	
leaves	
tannins: condensed tannins (leucoanthocyanidin, catechin units), and hydrolysable (ellagi-) tannins (Hegnauer, 2001);	
flavonoids: quercetin glycosides abundant (quercitrin, isoquercitrin, rutin, quercetin-3-glucuronide), kaempferol (kaempferol-3-glucoside) and luteolin glycosides also occur, frequently myricetin-3-glucoside (the pattern of flavonoids depends on species) (Hegnauer, 2001);	
hydroxycinnamic acids: caffeic acid, caffeoyl tartrate, mono-cinnamoyl tartrates, chlorogenic acid (Hegnauer, 2001);	
stilbenes: derivatives based on resveratrol act as phytoalexins (viniferins) with fungitoxic effects (only synthesised by infection with fungi and exposure to UV-light) (Hegnauer, 2001)	
young leaves	
cyanogenic compounds: partly in large amounts depending on the age of leaves and sort of *Vitis vinifera* (Hegnauer, 2001)	
fruits	
anthocyanins: cyanidin, paeonidin, delphinidin, petunidin, malvidin as aglyca (Hegnauer, 2001);	
flavonoids: kaempferol, myricetin and quercetin as aglyca; their occurrence is depending on sorts of fruits (Hegnauer, 2001);	
tannins: condensed tannins (catechin, epicatechin, epicatechingallat, gallocatechin units and dimeric procyanidins) (Hegnauer, 2001)	
roots	
tannins: condensed tannins (catechin units) (Hegnauer, 2001)	
wood	
stilbenes: di- and oligomeric compounds, based on resveratrol	
Vitis cinerea Engelm.	
leaves flavonoids: vitexin, isovitexin, orientin, isoorientin, isoquercitrin; free amino acids: large amounts of hydroxyprolin (Hegnauer, 2001)	tasted in spring, eaten in autumn
fruits	
anthocyanins (aglyca see above) exclusively as 3-monoglucosides (Hegnauer, 2001)	
unripe fruits	
free amino acids: large amounts of hydroxyprolin (Hegnauer, 2001)	
wood	tasted in spring, eaten in autumn
Vitis rotundifolia Michx.	
leaves	tasted in spring, eaten in autumn
fruits	
anthocyanins (aglyca see above) exclusively as 3,5-diglucosides (Hegnauer, 2001)	
wood	tasted in spring, eaten in autumn

remark: Condensed tannins indicated in Tab. II.2 are often proved to occur by testing for anthocyanidins: as the interflavan carbon-carbon bond of proanthocyanidins is labile to acidic conditions especially when heated (acid catalysed decompensation), catechin units and anthocyanidins (coloured products) are yielded. (Bate-Smith, 1954; Bate-Smith and Lerner, 1954; Bate-Smith, 1962; Haslam, 1975; Bate-Smith, 1977; Bate-Smith, 1978).

Under these reaction conditions flavonoids are hydrolysed yielding flavonoid aglyca. Hence it has to be emphasised that aglyca mentioned in Tab. II.2 did normally not occur genuinely in plant specimens. As most reports on hydroxycinnamic acids are based on leaf analyses of Bate-Smith (1962), their occurrence is mentioned although they are normally esterified and not in free state (Ibrahim and Barron, 1989).

Most cited work is the chemotaxonomical review given by Hegnauer (2001). In case of leguminous species his last three volumes are cited explicitly, in case of the other ten volumes, it was relinquished to cite all respective volumes.



glc: glucopyranose rha: rhamnopyranose
fuc: fucopyranose ara (f): arabinofuranose
xyl: xylopyranose qui: quinovopyranose

Fig. II.1: Julibroside III is depicted as representative saponin of Albiziae cortex.

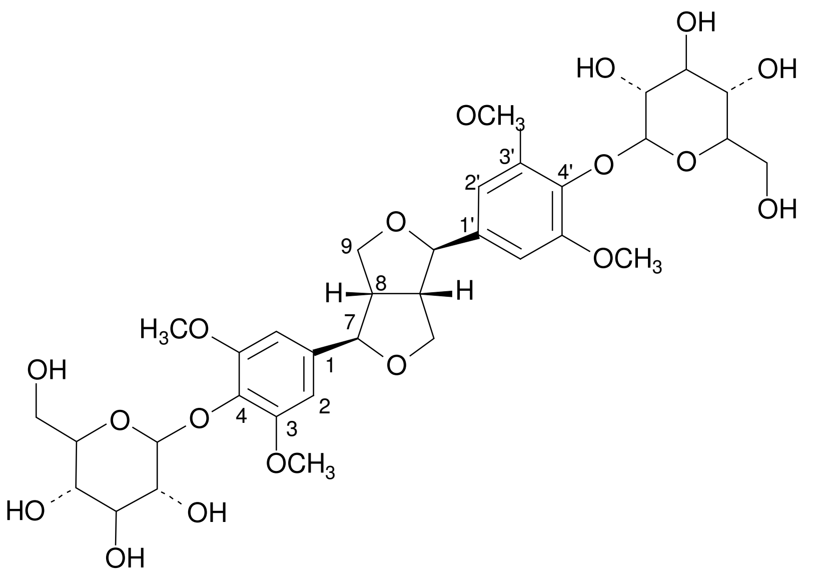


Fig. II.2: Syringaresinoldiglucoside (syn. (-)-syringaresinol-4,4’-bis-O-ß-D-glucopyranoside; (-)-syringaresinol-di-ß-D-glucoside), which occurs in Albiziae cortex and in *Liriodendron tulipifera*.

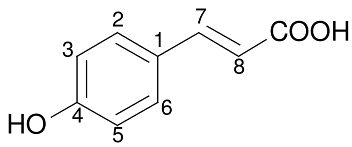
II.3 Elucidation of compounds isolated from leaves of *A. julibrissin* and *R. copallina*

Compounds, whose structure could be conclusively elucidated, are in alphabetical order. UV-data associated with analytical HPLC analyses, and results of different NMR-spectroscopic and mass-spectrometric experiments are presented, respectively. Compounds of fractions, for which the chemical structure could not be conclusively elucidated due to a lack of informative NMR data are documented according to their numbering of fractions. For these fractions UV and mass data are presented. If possible, the chemical formula calculated from the mass data together with a description of the chemical constituents are given. Fractions eluted from the polyamide column that were not further separated on the HPLC column are merely described by their UV data. The numbering of conclusively elucidated compounds and additional fractions follows their alphabetical and numerical order. (Some details given in this appendix are discussed in chapter C.III).

1 p-Coumaric acid

Fractions	A18, A22	Formula	$C_9H_8O_3$
IUPAC-Name	2-propenoic acid, 3-(4-hydroxyphenyl)-	MW	164
INN-Names	4-hydroxycinnamic acid; trans-p-hydroxycinnamic acid; trans-(4-hydroxy)-cinnamic acid; p-coumaric acid	Cas. No.	[501-98-4]

Structure



Hydroxycinnamic acids often build conjugated forms and seldom occur in the free state (Ibrahim and Barron, 1989). Hence, the question arises, as to whether p-coumaric acid found by HPLC analysis is an artefact due to the extraction procedure, or whether it is genuinely occurring in the plant?

HPLC

mean retention time	UV-Data λ_{max} / nm		
24.4 min.	226	sh 300	309
Ibrahim and Barron (1989) (MeOH/EtOH)		sh 295	312

EI-MS (A18)

m/z 164	(100 %)	$C_9H_8O_3$:		$[M]^{+\bullet}$
m/z 147	(36 %)	$C_9H_7O_2$:	$- OH^{\bullet}\rightarrow$	$[M - OH]^+$
m/z 119	(24 %)	C_8H_7O:		$[M - OH - CO]^+$
m/z 118	(16 %)	C_8H_6O:	$- H^{\bullet}\rightarrow$	$[M - OH - CO - H]^{+\bullet}$
m/z 91	(19 %)	C_7H_7:		$[M - OH - CO - CO]^+$, benzyl cation $\leftrightarrow$ tropylium ion
m/z 65	(13 %)	C_5H_5:		$[M - OH- CO - CO - C_2H_2]^+$

Search in NIST MS database verified the identification as trans-(4-hydroxy)-cinnamic acid.

Characteristic ions given by Bocchi *et al.* (1996) [m/z 164 (100 %), m/z 147 (41 %) and m/z 119 (26 %)] agree well with findings of this study.

ESI-MS (A18)

negative mode:

m/z 163 (100 %) : $[M - H]^-$

Only the negative mode was used yielding valuable information. As reference, see Gioacchini *et al.* (1996).

ESI-MS (A22)

positive mode:

m/z 371 (100 %)

m/z 209 (67 %) : $[Na\ salt + Na]^+$

MS/MS of mass 371

m/z 371 (9 %)

m/z 287 (100 %) : $[371 - DMSO\text{-}d_6]^+$

negative mode:

m/z 349 (83 %) : $[2M - 2H + Na]^-$

m/z 163 (100 %) : $[M - H]^-$

At least six other compounds were detected in the HPLC chromatogram in addition, although no definite structure or molecular weight could be assigned. However, HPLC, UV- and mass spectra indicate p-coumaric acid to occur in fraction A22.

^{1}H-NMR-Parameters

Assignment	No. of H	δ [ppm] multiplicity J [Hz] A18 (DMSO-d_6)	 Ibrahim and Barron, 1989 (MeOD; DMSO-d_6)
C2-H / C6-H	2	7.51 AA'XX' N = 8.7	7.45-7.56, d 8.0-8.8
C7-H	1	7.48 d 15.8	7.43-7.81, d 16
C3-H / C5-H	2	6.78 AA'XX' N = 8.7	6.79-6.84, d 8.0-8.8
C8-H	1	6.28 d 15.8	6.15-6.64, d 16

Ibrahim and Barron (1989) have discussed the effect of Z,E- isomerism of cinnamic acids on ^{1}H-NMR-spectroscopy. All values given in the table above are valid for deutero-methanol and DMSO, the most common solvents. The E-cinnamoyl residue is detectable by a pair of doublets with shift values of 6.15-6.64 ppm for C8-H and 7.43-7.81 ppm for C7-H, the corresponding coupling constant is about 16 Hz, which agrees well with findings of this study. In the rarely occurring Z-isomeric form, the signals for C8-H and C7-H are moved upfield to 5.87-5.99 and 6.84-6.95 ppm, respectively. The coupling constant is lowered to roughly 13 Hz. Therefore, proton spectroscopy is an appropriate method to distinguish between both isomeric forms.

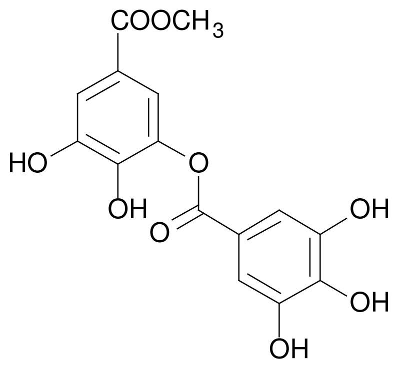
In addition, the p-hydroxyl substitution and the E-isomeric form in case of an o-hydroxyl substitution, occurs normally in the Z-form, which results in a spontaneous cyclisation yet yields the chemical class of coumarins. The o-hydroxycinnamic acid is the precursor of the class of coumarins.

In this study, there was not sufficient substance isolated in order to perform a ^{13}C-NMR measurement. Reference values for ^{13}C-spectroscopy are given in Ternai and Markham (1976), and Ibrahim and Barron (1989).

2 p- and m-Digallic acid methyl ester

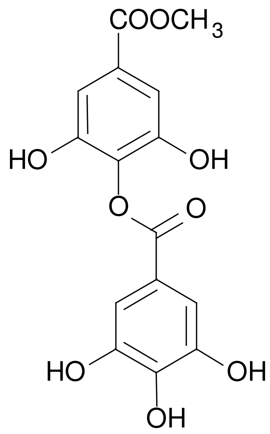
Fractions	R16	Formula	$C_{15}H_{12}O_9$
INN-Names	p- and m-digallic acid methyl ester p- and m-methyl digallate	MW	336

Structure



m-I

m-II



p-I

p-II

p-I; m-I: gallic acid methyl ester moiety of the corresponding para or meta substituted compound
p-II; m-II: gallic acid moiety in para or meta position of the gallic acid methyl ester moiety

HPLC

mean retention time	UV-Data λ_{max} / nm
23.8 min. (p-isomer of methyl digallate)	214 sh 250 280
26.3 min. (m-isomer of methyl digallate)	216 272

EI-MS

m/z 184 (30 %) $C_8H_7O_5$: $[M]^{+\bullet}$, gallic acid methyl ester
m/z 153 (100 %) $C_7H_5O_4$: $- OCH_3^{\bullet} \rightarrow$ $[M - OCH_3]^+$
m/z 125 (19 %) $C_6H_5O_3$: $[M - OCH_3 - CO]^+$, pyrogallol cation

Both of the dimeric substances consist of a gallic acid and a gallic acid methyl ester moiety. Due to the high polarity of these compounds, thermolysis commenced under EI-MS conditions. Hence, the only detectable molecule radical cation and subsequent fragmentation products originated from gallic acid methyl ester. In the gallic acid methyl ester itself (see there), m/z 153 is the base peak. The molecule radical cation m/z 336 was not detectable in the EI spectrum. During the procedure of structure elucidation, the information gained by EI-MS was not conclusive. Therefore, the following mass spectrometric investigations were undertaken in addition.

FAB-MS

negative mode:

m/z 335 (100 %) : $[M - H]^-$

DCI-MS

positive mode:

m/z 354 (41 %) : $[M + NH_4]^+$
m/z 219 (19 %) : $[\text{gallic acid methyl ester moiety} + NH_4 \bullet NH_3]^+$
m/z 202 (100 %) : $[\text{gallic acid methyl ester moiety} + NH_4]^+$

negative mode:

m/z 336 (54 %)	:	$[M]^{-\bullet}$
m/z 152 (100 %)	:	$[C_7H_4O_4]^{-\bullet}$, a chinoid keten

Under DCI-MS conditions in the positive mode, the molecule of interest is generally detectable as a so-called quasimolecular ion, i.e. the analyte molecule which is either attached to a reactant gas cation, or occasionally to a cluster ion of the reactant gas. On the contrary, in the negative mode, molecule radical anions may also occur. Polar compounds like the dimeric isomers of the R16, soon experience reactions of pyrolysis. The radical anion m/z 152 is such a product of pyrolysis and even builds the base peak of that spectrum.

^{1}H-NMR-Parameters

Signal	Assignment	No. of H	δ [ppm] multiplicity J [Hz] R16 (DMSO-d_6)
A	-OH	10[a]	9.85 s, br.
B	-OH		9.40 s, br.
C	m-I/ C2-H	1	7.34 d 2.1
D	m-I/ C6-H	1	7.17 d 2.1
E	m-II/ C2/6-H	2	7.10 s
F	p-II/ C2/6-H	2	7.09 s
G	p-I/ C2/6-H	2	7.04 s
H	p/ OCH_3	3	3.81 s
I	m/ OCH_3	3	3.78 s

[a] The signals A and B are not completely resolved in the spectrum, representing a total of 10 hydroxyl groups.

^{13}C-NMR-Parameters

Signal	Assignment	δ [ppm] multiplicity J [Hz] R16 (DMSO-d_6)	Nishizawa *et al.*, 1982 (acetone-d_6)
a	p-I/ COO	166.8	166.8
b	m-I/ COO	166.6	166.6
c	m-II/ COO	165.0	164.9
d	p-II/ COO	164.3	164.2
e	p-I/ C3/ C5	151.4	151.2
f	m-I/ C3	147.3	143.3[a]
g	m-II/ C3/5	146.6	146.0
h	p-II/ C3/5	146.5	145.9
i	m-I/ C4	143.8	139.4
j	m-II/ C4	140.0	139.7
k	p-II/ C4	139.8	138.7[b]
l	m-I/ C5	139.8	146.9
m	p-I/ C4	132.0	132.4
n	p-I/ C1	127.7	128.7
o	m-I/ C1	120.2	120.8[c]
p	p-II/ C1	119.3	120.7[d]
q	m-II/ C1	119.1	121.6
r	m-I/ C6	116.5	114.5
s	m-I/ C2	114.4	117.3
t	p-II/ C2/6	110.2	110.8[d]
u	m-II/ C2/6	110.2	110.7

Signal	Assignment	δ [ppm] multiplicity J [Hz] R16 (DMSO-d_6)	Nishizawa *et al.*, 1982 (acetone-d_6)
v	p-I/ C2/6	108.9	109.8
w	p-I/ $-OCH_3$	52.1	52.4
x	m-I/ $-OCH_3$	51.9	52.2

[a-d] Regarding the reference values given by Nishizawa *et al.* (1982), there are some discrepancies between his depicted spectra and the values of his experimental part. Please note the following demarcations:

[a] not assigned, only listed in the spectrum.

[b] This shift is reported in his experimental part, whereas it is identified as 139.3 ppm in his depicted spectrum.

[c] This signal is assigned to the m-isomer in the experimental part, but to the p-isomer in his depicted spectrum.

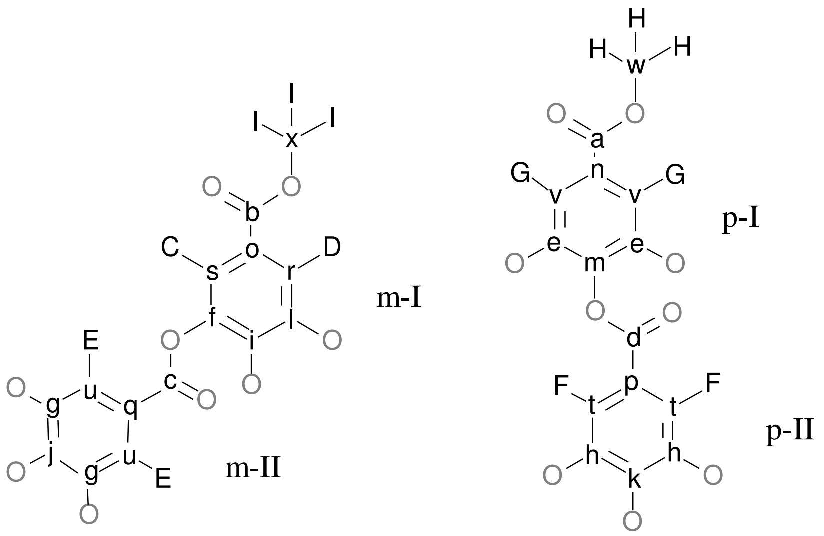
[d] These signals are assigned to the p-isomer in the experimental part, but to the m-isomer in the depicted spectrum.

Judging from the signal intensities, assignments of this study are correct as given in the table. The DEPT experiment was performed to reassure the assignment.

HMBC

Tab. II.3: Heterocorrelation between the signals of the ^{1}H and ^{13}C spectrum.

^{13}C / ^{1}H	COO				C3/5				C4			C5	C4	C1				C2/6					CH_3	
	a	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**	**k**	**l**	**m**	**n**	**o**	**p**	**q**	**r**	**s**	**t**	**u**	**v**	**w**	**x**
C		X				X			X			X			X			X						
D		X				X			X			X			X				X					
E			X				X			X							X				X			
F				X				X			X					X				X				
G	X				X								X	X								X		
H	X																							
I		X																						



m-methyl digallate p-methyl digallate

Fig. II.3: Characters (capitals indicate protons, small letters the carbon atoms) are used for assignments gained by two-dimensional experiments.

The HMBC-spectrum (Tab. II.3) yields the correlation of assignments as shown in Fig. II.3. Critical assignments are as follows:

Signals C/D. Shift increments of -OH and -OCOPh predict a downfield signal for C2-H (Pretsch *et al.*, 1990).

Signals r/s. In HMBC, r does not correlate to D and s not to C. Hence, proton D must be bound to carbon r and proton C to carbon s.

Signals f/l. In HMBC, f correlates stronger to C than to D, while for l holds the inverse. It is assumed that two-bond couplings in the phenyl-residue are stronger than four-bond couplings (Hansen and Jakobsen, 1975).

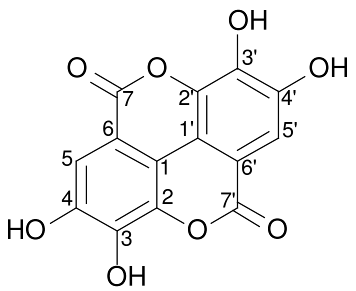
Signals t/u. These signals were not resolved by the spectrometer in this study. Therefore, the assignment might be exchanged.

Signals of the units m-II and p-II. The carbon shifts of these two units are similar in pairs. The assignment within a given pair was made using the experimental spectrum of a synthetic mixture of methyl m- and p-digallate reported by Nishizawa *et al.* (1982). There, the components could easily be identified by signal intensities. The proton signals were then correlated by HMBC in this study.

3 Ellagic acid

Fractions	R21	Formula	$C_{14}H_6O_8$
INN-Names	ellagic acid	MW	302

Structure



HPLC

mean retention time	UV-Data λ_{max} / nm			
26.6 min.	253	sh 304	sh 352	367
Khac *et al.*, 1990 (EtOH)	254			365
Nawwar *et al.*, 1994 (MeOH)	255			362
Hussein *et al.*, 1997 (MeOH)	255			362

EI-MS

m/z 302 (4%) $C_{14}H_6O_8$: $[M]^{+\bullet}$

Impurities:

m/z 184 (61 %) $C_8H_8O_5$: $[\text{gallic acid methyl ester}]^{+\bullet}$

m/z 170 (6 %) $C_7H_6O_5$: $[\text{gallic acid}]^{+\bullet}$

m/z 153 (100 %) $C_7H_5O_4$: $- OH^{\bullet} \rightarrow$ $[\text{gallic acid - OH}]^{+}$

$- OCH_3^{\bullet} \rightarrow$ $[\text{gallic acid methyl ester - } OCH_3]^{+}$

m/z 126 (48 %) $C_6H_5O_2$: $[\text{gallic acid - } CO_2]^{+\bullet}$

m/z 108 (16 %) $C_6H_4O_2$: $[\text{gallic acid - } CO_2\text{- } H_2O]^{+\bullet}$

m/z 79 (16 %) C_5H_3O: $[\text{gallic acid - OH- CO - } H_2O \text{ - CO}]^{+}$;

$[\text{gallic acid methyl ester - } OCH_3\text{- CO - } H_2O \text{ - CO}]^{+}$

As a reference for EI-MS, see Khac *et al.* (1990): $[M]^{+\bullet}$: m/z 302.
Ellagic acid, an artefact of the hexahydroxydiphenic acid and is usually generated through the isolation procedure, is a highly symmetrical molecule. The only valuable clue to this compound in the mass spectrum is m/z 302, the molecule radical cation. A spectrum taken from the library (NIST MS) corroborates that there is no special pattern of fragmentation of ellagic acid, and if any fragmentation occurs, it is to a lesser extent. Hence, the base peak is normally the molecule radical cation. Due to impurities in fraction R21, especially of gallic acid methyl ester, the molecule radical ion of ellagic acid just amounts to about 4 %.

^{1}H-NMR-Parameters

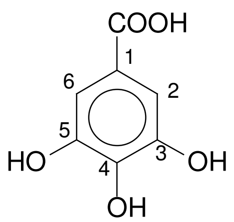
		δ [ppm] multiplicity J [Hz]		
Assignment	No. of H	R21 (DMSO-d_6)	Khac *et al.*, 1990 (DMSO-d_6)	Nawwar *et al.*, 1994 (DMSO-d_6)
C5-H, C5'-H	2	7.42 s	7.50 s	7.5 s

Ellagic acid only gives one signal due to its highly symmetrical structure.

4 Gallic acid

Fractions	A8, R3	Formula	$C_7H_6O_5$
IUPAC-Name	benzoic acid, 3,4,5-trihydroxy-	MW	170
INN-Names	gallic acid	Cas. No.	[149-91-7]

Structure



HPLC

mean retention time	UV-Data λ_{max} / nm
11.9 min.	215 271
Nawwar *et al.*, 1994 (MeOH)	272
Hussein *et al.*, 1997 (MeOH)	272

EI-MS

m/z 170	(100 %)	$C_7H_6O_5$:		$[M]^{+\bullet}$
m/z 153	(66 %)	$C_7H_5O_4$:	$- OH^{\bullet} \rightarrow$	$[M - OH]^+$
m/z 135	(7 %)	$C_7H_3O_3$:		$[M - OH - H_2O]^+$
m/z 125	(17 %)	$C_6H_5O_3$:		$[M - OH - CO]^+$, pyrogallol cation
m/z 107	(4 %)	$C_6H_3O_2$:		$[M - OH - CO - H_2O]^+$
m/z 79	(9%)	C_5H_3O:		$[M - OH - CO - H_2O - CO]^+$
m/z 53	(20%)	C_3HO:		$[M - OH - CO - H_2O - CO - C_2H_2]^+$

The relative abundance is given as a mean of both fractions (A8 and R3).
Characteristic ions and their abundance given by Bocchi *et al.* (1996) are in accordance with those detected in this study [m/z 170 (100 %); m/z 153 (81 %)].

ESI-MS

negative mode:

m/z 169	(73 %)	:	$[M - H]^-$
m/z 125	(100%)	:	$[M - OH - CO - H]^-$, $[pyrogallol - H]^-$

As reference see Gioacchini *et al.* (1996).

NMR-Data

In the following, fraction R3 is documented as representative sample.

^{1}H-NMR-Parameters

		δ [ppm] multiplicity J [Hz]	
Assignment	No. of H	R3 (DMSO-d_6)	Nawwar *et al.*, 1982 and 1984a (DMSO-d_6)
C3,4,5-OH	3	9.2 s, br.	
C2-H, C6-H	2	6.92 s	6.98 s

The three hydroxyl groups at C3, C4 and C5 yield a highly broad singlet, due to the acidity of their protons. It is reasonable to assume a fast proton exchange is taking place. When broad signals occur, the corresponding integrals are sometimes smaller than expected.

^{13}C-NMR-Parameters

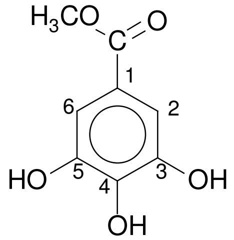
	δ [ppm] multiplicity J [Hz]	
Assignment	R3 (DMSO-d_6)	Nawwar *et al.*, 1982 and 1994 (DMSO-d_6)
- COO	167.5	167.7
C3/5	145.4	145.5
C4	138.0	138.1
C1	120.5	120.6
C2/6	108.7	108.8

DEPT experiment: A positive signal at 108.7 ppm verifies the position of both tertiary carbon atoms C2 and C6.

5 Gallic acid methyl ester

Fractions	R1, R6, R15, R20, R22, R23	Formula	$C_8H_8O_5$
IUPAC-Name	Benzoic acid, 3,4,5-trihydroxy methyl ester	MW	184
INN-Names	gallic acid methyl ester methyl gallate	Cas. No.	[99-24-1]

Structure



HPLC

mean retention time	UV-Data λ_{max} / nm	
18.2 min.	216	272
Parmar *et al.*, 1997 (MeOH)	218	272

EI-MS

m/z 184	(63 %)	$C_8H_8O_5$:	$[M]^{+\bullet}$
m/z 153	(100 %)	$C_7H_5O_4$: - $OCH_3^{\bullet}$ →	$[M - OCH_3]^+$
m/z 125	(23 %)	$C_6H_5O_3$:	$[M - OCH_3 - CO]^+$, pyrogallol cation
m/z 107	(4 %)	$C_6H_3O_2$:	$[M - OCH_3 - CO - H_2O]^+$
m/z 79	(7 %)	C_5H_3O:	$[M - OCH_3 - CO - H_2O - CO]^+$

The relative abundance is given as a mean of all fractions.
Depending on the temperature, the occurrence of the molecule radical cation in the mass spectrum varies between 50 % and 100 % relative abundance. The $[M - OCH_3]^+$ ion, m/z 153, forms the base peak, while m/z 125 yields a mean of 23 % relative abundance, m/z 107 a mean of 4 % and m/z 79 a mean of 7 %, respectively. This fragmentation pattern is also in accordance with data from the NIST MS data base. Fragmentation resembles that of gallic acid.

^{1}H-NMR-Parameters

		δ [ppm] multiplicity J [Hz]	
Assignment	No. of H	average of 6 fractions (DMSO-d_6)	Parmar *et al.*, 1997 (DMSO-d_6)
C3,4,5-OH	3	9.15 s, br.	8.93 s, br.
C2-H, C6-H	2	7.00 s	7.00 s
$-OCH_3$	3	3.74 s	3.81 s

As for the gallic acid, the signal for the three hydroxyl groups is very broad because of the acidity of the corresponding protons. Integration of these signals involves the same difficulties as mentioned for the gallic acid.
The averaged values of all fractions containing gallic acid methyl ester (R1, R6, R15, R20, R22 and R23) are represented as ^{1}H-NMR-parameters.

^{13}C-NMR-Parameters

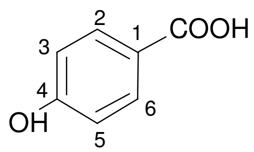
Assignment	δ [ppm] multiplicity J [Hz] R1 (DMSO-d_6)	Sakurai and Okuruma, 1983 (DMSO-d_6)
-COO	166.3	166.2
C3/5	145.6	145.4
C4	138.4	138.2
C1	119.4	119.2
C2/6	108.6	108.4
-OCH3	51.6	51.5

DEPT: The signals for the C2/6 and the -OCH_3 are visible.
The ^{13}C-NMR could only be performed with the R1, the isolated amount of all other fractions was too small for this experiment.

6 p-Hydroxy-benzoic acid

Fractions	A10	Formula	$C_7H_6O_3$
IUPAC-Name	Benzoic acid, 4-hydroxy-	MW	138
INN-Names	p-hydroxy-benzoic acid		

Structure



HPLC

mean retention time	UV-Data λ_{max} / nm
18.6 min.	255
Van Sumere, 1989 (MeOH)	252

The minimum value at 224 nm given by Van Sumere (1989) is the same as found in the spectrum gained in this study.

EI-MS

m/z 138	(85 %)	$C_7H_6O_3$:		$[M]^{+\bullet}$
m/z 121	(100 %)	$C_7H_5O_2$:	$- OH^{\bullet} \rightarrow$	$[M - OH]^+$
m/z 110	(19 %)	$C_6H_6O_2$:		$[M - CO]^{+\bullet}$
m/z 93	(41 %)	C_6H_5O:		$[M - OH- CO]^+$
m/z 65	(32 %)	C_5H_5:		$[M - OH- CO - CO]^+$, pentacyclodienyl cation

As expected for p-hydroxybenzoic acid, base peak is m/z 121, the $[M - OH]^+$ ion. The molecule radical cation m/z 138 occurs with 85 % relative abundance. The spectrum of an authentic sample was used as reference spectrum.
Characteristic ions given by Bocchi *et al.* (1996) are in accordance with values found in this study [m/z 138 (78 %); m/z 121 (100 %); m/z 93 (30 %)].

^{1}H-NMR-Parameters

Assignment	No. of H	δ [ppm] multiplicity J [Hz] A10 (DMSO-d_6)	authentic sample (DMSO-d_6)
- COOH	1		12.4 s, br.
-OH	1		10.2 s, br.
C2-H, C6-H	2	7.78 m, AA'XX', N=8.7	7.82 m, AA'XX', N=8.7
C3-H, C5-H	2	6.82 m, AA'XX', N=8.7	6.85 m, AA'XX', N=8.7

Coupling constants were not explicitly calculated for the AA'XX'system. ($N = |J_{2,3} + J_{2,5}|$).

7 Kaempferol

The main compound of the fractions A17 and A24 (A24-1) is kaempferol, which is purported to be an artefact generated through the isolation process. This is corroborated by the finding that kaempferol aglyca were detected in two different fractions with a large gap of time between them. As a second compound in A24 (A24-2), a hydroxycinnamic acid derivative was found by mass spectrometry with a molecular weight of 340 Da. It is postulated to consist of a caffeic acid methyl ester (= 3,4-dihydroxycinnamic acid methyl ester) moiety and a p-coumaric acid (= p-hydroxycinnamic acid) moiety.

Fractions A17, A24 **(A24-1)**

IUPAC 4H-1-Benzopyran-4-one, 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-

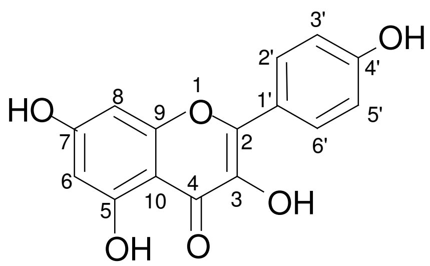
Formula $C_{15}H_{10}O_6$

MW 286

Cas. No. [520-18-3]

INN-Names **kaempferol**
3,4´,5,7-tetrahydroxyflavone

Structure



hydroxycinnamic acid derivative (A24-2)

proposed structure caffeic acid methyl ester + p-coumaric acid moiety.

Formula $C_{19}H_{16}O_6$

MW 340

HPLC

mean retention time	UV-Data λ_{max} / nm				
36.6 min. (kaempferol)	sh 245	265	sh 292	sh 320	365
Mabry *et al.*, 1970 (MeOH)	sh 253	266	sh 294	sh 322	367

The UV-spectrum and the retention time of the substance investigated was in accordance with data gained from an authentic sample (kaempferol purchased from Fluka), which was submitted to the same analytical conditions.

EI-MS (A17)

m/z 286	(100 %)	$C_{15}H_{10}O_6$:	$[M]^{+\bullet}$, kaempferol
m/z 258	(5 %)	$C_{14}H_{10}O_5$:	$[M - CO]^{+\bullet}$
m/z 229	(6 %)	$C_{13}H_9O_4$:	$[M - CO - CO - H]^+$
m/z 153	(6 %)	$C_7H_5O_4$:	$[A_1 + H]^+$
m/z 121	(14 %)	$C_7H_5O_2$:	$[B_2]^+$
m/z 69	(5 %)		

The spectrum described, coincides with the spectrum of kaempferol given by the NIST database.

ESI-MS (A17)

negative mode:

m/z 571	(40 %)	:	$[2 M - H]^-$, dimer of kaempferol
m/z 285	(100 %)	:	$[M - H]^-$, kaempferol

From UV-data and mass spectrometry it is concluded that the substance under investigation is kaempferol. It can be assumed that free kaempferol is more likely to be an artefact through isolation procedure than to occur as such. This indicates two different natural compounds both containing kaempferol as aglycone moiety.

EI-MS (A24-2)

m/z 340	(78 %)	:	$[M]^{+\bullet}$, hydroxycinnamic acid derivative
m/z 177	(100 %)	$C_{10}H_9O_3$:	[caffeic acid methyl ester moiety]$^+$
m/z 164	(59 %)	$C_9H_8O_3$:	[p-coumaric acid]$^{+\bullet}$
m/z 161	(81 %)		

According to ion traces fragment m/z 177 and fragment m/z 161 are generated from the same original molecule.

ESI-MS (A24-2)

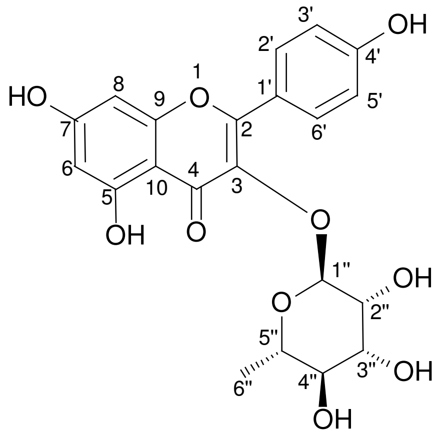
negative mode:

m/z 339	(100 %)	:	$[M - H]^-$
MS/MS of mass 339:			
m/z 339	(14 %)	:	$[M - H]^-$
m/z 163	(100 %)	:	[p-coumaric acid - H]$^-$

8 Kaempferol-3-O-α-L-rhamnopyranoside

Fractions	A14, R12	Formula	$C_{21}H_{20}O_{10}$
INN-Names	kaempferol-3-O-α-L-rhamnopyranoside, afzelin	MW	432

Structure



Appearance Pale yellowish powder (Fukunaga *et al.*, 1988), or yellow needles (Tanaka *et al.*, 1984).

HPLC

mean retention time	UV-Data λ_{max} / nm			
29.6 min.	263	sh 292	sh 315	342
Fukunaga *et al.*, 1988 (EtOH)	263			342
Tanaka *et al.*, 1984 (MeOH)	266			344

Mean values of both fractions are presented.
The values found for afzelin measured with diode array during the analytical HPLC analysis agree well with the data of Fukunaga *et al.* (1988).

EI-MS (R12)

m/z 286 (100 %) $C_{15}H_{10}O_6$: $[M]^{+\bullet}$, kaempferol (aglycone)
m/z 258 (7 %) $C_{14}H_{10}O_5$: $[M - CO]^{+\bullet}$
m/z 229 (6 %) $C_{13}H_9O_4$: $[M - CO - CO - H]^+$
m/z 153 (9 %) $C_7H_5O_4$: $[A_1 + H]^+$
m/z 121 (12 %) $C_7H_5O_2$: $[B_2]^+$
m/z 69 (10 %)
impurity:
m/z 302 (8 %) $C_{15}H_{10}O_7$: $[\text{quercetin}]^{+\bullet}$

ESI-MS (R12)

positive mode:
m/z 455 (100 %) : $[M + Na]^+$, kaempferol glycoside
m/z 287 (32 %) : $[\text{aglycone} + H]^+$, kaempferol
impurity:
m/z 603 (24 %) : $[M + Na]^+$

negative mode:

m/z 431	(82 %)	:	$[M - H]^-$ of kaempferol glycoside
m/z 285	(22 %)	:	$[aglycone - H]^-$
impurity:			
m/z 579	(9 %)	:	$[M - H]^-$
m/z 301	(100 %)	:	$[aglycone - H]^-$, quercetin

The molecular weight of the impurity is found to be 580 Da by complementary information gained from the positive and negative ESI-spectrum.

EI-MS (A14)

m/z 286	(100 %)	$C_{15}H_{10}O_6$:	$[M]^{+\bullet}$, kaempferol (aglycone)
m/z 258	(6 %)	$C_{14}H_{10}O_5$:	$[M - CO]^{+\bullet}$
m/z 229	(5 %)	$C_{13}H_9O_4$:	$[M - CO - CO - H]^+$
m/z 153	(4 %)	$C_7H_5O_4$:	$[A_1 + H]^+$
m/z 121	(9 %)	$C_7H_5O_2$:	$[B_2]^+$
m/z 128	(10 %)		
impurity:			
m/z 302	(3 %)	$C_{15}H_{10}O_7$:	$[quercetin]^{+\bullet}$

ESI-MS (A14)

positive mode:

m/z 471	(25 %)	:	$[M + K]^+$, kaempferol glycoside
m/z 455	(100 %)	:	$[M + Na]^+$, kaempferol glycoside
m/z 287	(86 %)	:	$[aglycone + H]^+$, kaempferol

negative mode:

m/z 431	(100 %)	:	$[M - H]^-$, kaempferol glycoside
m/z 285	(24 %)	:	$[aglycone - H]^-$, kaempferol
impurity:			
m/z 301	(76 %)	:	$[quercetin - H]^-$

Fragment m/z 153, which is detectable in the EI-spectrum and is corresponding to fragment $[A_1 + H]^+$, does not only occur for kaempferol aglycone during the retro-Diels Alder reaction with subsequent H radical transfer, but also for the impurity quercetin.
Data found in the EI-MS spectrum corresponded well with those given by Tanaka *et al.* (1984).

NMR-Data

Fraction R12 was used as representative spectrum for both, the ^{1}H and ^{13}C experiments.

^{1}H-NMR-Parameters

Assignment	No. of H	δ [ppm] multiplicity J [Hz] R12 (DMSO-d_6)	Fukunaga *et al.*, 1988[a] (DMSO-d_6)	Tanaka *et al.*, 1984[b] (DMSO-d_6)
C5-OH	1	12.62 s		
C2'-H / C6'-H	2	7.75 AA'XX' N = 8.8	7.76 d 8.8	7.73 d 9
C3'-H / C5'-H	2	6.91 AA'XX' N = 8.8	6.92 d 8.8	6.93 d 9
C8-H	1	6.41 d 2.1	6.42 d 2.0	6.46 d 2
C6-H	1	6.21 d 2.1	6.22 d 2.0	6.24 d 2
C1" -H	1	5.29 d 1.5	5.30 d 1.5	5.30 d 2
C2" -H	1	4.25 - 4.00		
C3" -H	1	4.00 - 3.96		
C4" -H	1	3.66		
C5" -H	1	3.34		3.0-4.1, m (4 H)
C6" -H_3	3	0.79 d 5.8	0.80 d 5.8	0.82 d 4

[a] Except for the anomeric proton of the rhamnose moiety at C1", no assignments were presented by Fukunaga *et al.* (1988).

[b] No assignments were presented by Tanaka *et al.* (1984).

For proton shift values of the kaempferol aglycone and 3-O-α-L-rhamnosides as sugar moiety see Markham and Geiger (1994). Furthermore, quercitrin was used as example of this study to compare shift values. For a discussion on ^{1}H shift values in flavonol glycosides see chapter C.III. For measurements in acetone-d_6 see Matthes *et al.* (1980).

^{13}C-NMR-Parameters

Assignment	δ [ppm] multiplicity J [Hz] R12 (DMSO-d_6)	Fukunaga *et al.*, 1988 (DMSO-d_6)
C4	177.6	177.6
C7	164.1	164.2
C5	161.2	161.2
C4'	159.9	159.9
C2	157.1	157.1
C9	156.4	156.4
C3	134.1	134.2
C2'/C6'	130.5	130.5
C1'	120.4	120.5
C3'/ C5'	115.3	115.3
C10	104.0	104.1
C1"	101.7	101.7
C6	98.6	98.7
C8	93.6	93.7
C4"	71.0	71.1
C5"	70.5	70.0[a]
C3"	70.2	70.5[a]
C2"	70.0	70.3[a]
C6"	17.3	17.4

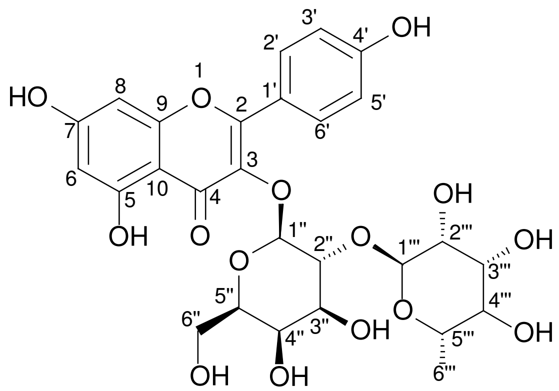
[a] Assignments bearing the same superscript may be reversed (Fukunaga *et al.*, 1988).

For synthesis of afzelin see Vermes *et al.* (1976).
For acetone-d_6 as solvent see Matthes *et al.* (1980).
DEPT: Positions of the tertiary carbon atoms were verified by the DEPT experiment.

9 Kaempferol-3-O-α-L-rhamnopyranosyl(1→2)ß-D-galactopyranoside

Fractions	A4	Formula	$C_{27}H_{30}O_{15}$
INN-Names	kaempferol-3-O-α-L-rhamnopyranosyl (1→2)ß-D-galactopyranoside	MW	594

Structure



Appearance Pale yellow needles (Yasukawa and Takido, 1987).

HPLC

mean retention time	UV-Data λ $_{max}$ / nm			
25.2 min.	265	sh 293	sh 318 (weak)	348
Yasukawa and Takido, 1987 (EtOH)	262	sh 300		350

EI-MS

m/z 286 (100 %) $C_{15}H_{10}O_6$: $[M]^{+\bullet}$, kaempferol (aglycone)
m/z 258 (7 %) $C_{14}H_{10}O_5$: $[M - CO]^{+\bullet}$
m/z 229 (6 %) $C_{13}H_9O_4$: $[M - CO - CO - H]^+$
m/z 153 (4 %) $C_7H_5O_4$: $[A_1 + H]^+$
m/z 121 (12 %) $C_7H_5O_2$: $[B_2]^+$
m/z 213 (4 %)
m/z 129 (7 %)
m/z 85 (11 %)

ESI-MS

positive mode:
m/z 633 (6 %) : $[M + K]^+$, kaempferol glycoside
m/z 617 (100 %) : $[M + Na]^+$, kaempferol glycoside
m/z 287 (17 %) : $[\text{aglycone} + H]^+$, kaempferol
negative mode:
m/z 593 (100 %) : $[M - H]^-$, kaempferol glycoside

^{1}H-NMR-Parameters

Assignment	No. of H	δ [ppm] multiplicity J [Hz] A4 (DMSO-d_6)	Yasukawa and Takido, 1987 (DMSO-d_6)
C5-OH	1	12.67 s	12.55
C2'-H / C6'-H	2	8.07 <u>AA'</u>XX' N = 8.9	8.07 d 8.3
C3'-H / C5'-H	2	6.85 AA'<u>XX'</u> N = 8.9	6.85 d 8.3
C8-H	1	6.42 d 2.0	6.39 d 1.9
C6-H	1	6.19 d 2.0	6.17 d 1.9
C1"-H (gal)	1	5.64 d 7.7	5.65 d 7.3
C1'''-H (rha)	1	5.05 ca. d 2[a]	5.04 s
C6'''-H_3	3	0.73 d 6.2	0.73 d 5.4

[a] signals were not completely resolved

^{13}C-NMR-Parameters (100 MHz)

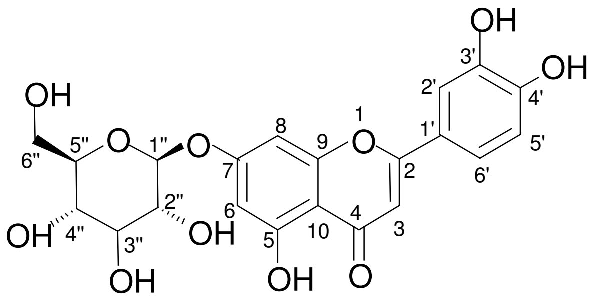
Assignment	δ [ppm] multiplicity J [Hz] A4 (DMSO-d_6)	Yasukawa and Takido, 1987 (DMSO-d_6)
C4	177.3	177.5
C7	164.1	164.8
C5	161.1	
C4'	159.8	159.8
C2	156.2	156.5
C9	155.9	156.0
C3	132.6	132.9
C2' / C6'	130.7	130.6
C1'	120.8	121.2
C3' / C5'	115.0	115.1
C10	103.8	104.0
C1" (gal)	98.6	99.1
C6	98.6	98.9
C8	93.5	93.7
C4"	68.1	68.2
C5"	75.5	75.9
C3"	73.9	74.1
C2"	75.0	75.6
C6"	60.1	60.4
C1''' (rha)	100.5	100.7
C2'''	70.6	70.9
C3'''	70.5	70.8
C4'''	71.8	72.3
C5'''	68.4	68.7
C6'''	17.1	17.2

C1" describes the anomeric proton of the galactose, C1''' that of the rhamnose moiety. For ^{1}H and ^{13}C shift values of flavonol glycosides of the kaempferol-rhamnopyranosyl-galactopyranoside type with additional sugar residues see Hasan *et al.* (1996).

10 Luteolin-7-O-ß-D-glucopyranoside

Fractions	R10, R11 (The main component of fraction R11 is quercitrin.)	Formula	$C_{21}H_{20}O_{11}$
		MW	448
INN-Names	luteolin-7-O-ß-D-glucopyranoside	Cas. No.	[5373-11-5]

Structure



TLC: yellow colour with diphenylboric acid aminoethyl ester spray (Pieroni *et al.*, 1996)

Appearance: yellow micro-needles, mp 240-245°C (Yoshizaki *et al.*, 1987); yellow-orange powder (Pieroni *et al.*, 1996)

HPLC

mean retention time	UV-Data λ_{max} / nm			
25.8 min.	254	sh 267		348
Nawwar *et al.*, 1989 (MeOH)	255	267		346
Mabry *et al.*, 1970 (MeOH)	255	sh 267		348
Kaneta *et al.*, 1980 (MeOH)	255	sh 267		348
Pieroni *et al.*, 1996 (MeOH)	255	sh 265		349
Impurities: 25.4 min. (R10)	sh 254	261	sh 297	349
27.5 min. (R11) [quercitrin, main compound of R11]	255	sh 265	sh 301	349
27.7 min. (R11) [(presumably an apigenin derivative, see also Mabry *et al.* (1970)]	266			338

EI-MS (R10)

m/z 286	(100 %)	$C_{15}H_{10}O_6$:	$[M]^{+\bullet}$, luteolin (aglycone)
m/z 258	(11 %)	$C_{14}H_{10}O_5$:	$[M - CO]^{+\bullet}$
m/z 153	(28 %)	$C_7H_5O_4$:	$[A_1 + H]^+$
m/z 138	(7 %)		
m/z 134	(8 %)	:	$[B_1]^{+\bullet}$ of kaempferol
m/z 121	(14 %)	:	$[B_2]^+$ of kaempferol and / or apigenin
m/z 107	(12 %)		
m/z 73	(16 %)		
m/z 60	(21 %)		

Impurities in fraction R10 are the following aglyca:

m/z 318	(6 %)	:	$[M]^{+\bullet}$, myricetin
m/z 302	(1 %)	:	$[M]^{+\bullet}$, quercetin
m/z 270	(9 %)	:	$[M]^{+\bullet}$, apigenin

ESI-MS

positive mode:

m/z 449	(51 %)	:	$[M + H]^+$, luteolin-7-glucoside
m/z 471	(34 %)	:	$[M + Na]^+$
m/z 287	(85 %)	:	$[luteolin + H]^+$, aglycone
impurity:			
m/z 593	(100%)	:	$[M + Na]^+$

negative mode:

m/z 447	(100 %)	:	$[M - H]^-$, luteolin-7-glucoside
m/z 285	(6 %)	:	$[luteolin - H]^-$, aglycone
impurity:			
m/z 569	(19 %)	:	$[M - H]^-$

Ref.: EI-MS; Kaneta *et al.* (1980)

From complementary information of both the positive and negative ESI-mode, it is presumed that the unknown substance (impurity) has a molecular weight of 570.

EI-MS (R11)

m/z 286	(27 %)	$C_{15}H_{10}O_6$:	$[M]^{+\bullet}$, luteolin (aglycone)
Impurities:			
m/z 302	(100 %), main compound of this fraction		
m/z 270	(48 %)	$C_{15}H_{10}O_5$:	$[apigenin]^{+\bullet}$, presumably
m/z 242	(6 %)	$C_{14}H_{10}O_4$:	$[apigenin - CO]^{+\bullet}$, presumably
m/z 184	(11 %)	$C_8H_8O_5$:	$[gallic\ acid\ methyl\ ester]^{+\bullet}$
m/z 153	(34 %)	$C_7H_5O_4$:	$[gallic\ acid\ methyl\ ester - OCH_3]^+$
m/z 107	(14 %)	$C_6H_3O_2$:	$[gallic\ acid\ methyl\ ester - OCH_3 - CO - H_2O]^+$

The main compound of fraction R11 is quercitrin according to the UV and NMR spectra. The corresponding aglycone quercetin builds the base peak of the mass spectrum. It is reasonable to assume that the molecule radical cation m/z 270 is caused by apigenin which is corroborated by the corresponding UV spectrum. Apart from the two flavones and the single flavonol in the mass spectrum of fraction R11, the occurrence of gallic acid methyl ester can be assumed, especially due to its typical fragmentation pattern. Although fragment m/z 153 could also be interpreted as $[A_1 + H]^+$ fragment resulting from flavone / flavonol cleavage and subsequent $H^\bullet$ transfer, it is more likely to originate from gallic acid methyl ester fragmentation, the more as other characteristic fragments of flavones/ flavonols are lacking (see Mabry and Markham, 1975; and Fig. C.7, p. 102).

ESI-MS (R11)

positive mode:

m/z 471	(100 %)	:	$[M + Na]^+$
m/z 487	(41 %)	:	$[M + K]^+$
m/z 303	(54 %)	:	$[quercetin + H]^+$

As the molecular weight of quercitrin (quercetin-3-O-α-L-rhamnopyranoside) and of luteolin-7-O-ß-D-glucopyranoside is the same, both molecule cations $[M + Na]^+$ and $[M + K]^+$ may originate from either compound. However, the aglycone detected refers to quercetin.

negative mode:

m/z 447	(100 %)	:	$[M - H]^-$
m/z 431	(4 %)	:	$[apigenin - glucoside - H]^-$, presumably
m/z 301	(13 %)	:	$[quercetin - H]^-$

Again, the molecule ion [M] may derive from quercitrin or luteolin-7-O-glucoside.

References:
Nawwar *et al.* (1989); FAB-MS, positive mode of luteolin-7-O-ß-D-glucopyranoside:
m/z 449 : $[M + H]^+$
Pieroni *et al.* (1996); FAB-MS, positive mode:
m/z 449 : $[M + H]^+$
m/z 287 : $[M + H - glucose]^+$

^{1}H-NMR-Parameters

Assignment	No. of H	δ [ppm] multiplicity J [Hz] R10 (DMSO-d_6)	Markham and Geiger, 1994 (DMSO-d_6)	Nawwar *et al.*, 1989 (DMSO-d_6)	Pieroni *et al.*, 1996 (DMSO-d_6)
C5-OH	1	12.99 s	12.95 br.		
C6'-H	1	7.45 dd 8.4, 2.4	7.45 dd 8.1, 2.1		7.41 dd 9.1, 2.0
C2'-H	1	7.42 d 2.4	7.43 d 2.1	7.45 dd 8, 2.5 (C2'-H / C6'-H)	7.40 d 1.9
C5'-H	1	6.91 d 8.4	6.92 d 8.2	6.94 d 8	6.90 dd 8.1, 1.8
C8-H	1	6.80 d 2.2	6.79 d 2.1	6.81 d 2.5	6.54 d 2.1
C3-H	1	6.75 s	6.75 s	6.88 s	6.64 s
C6-H	1	6.45 d 2.2	6.45 d 2.1	6.44 d 2.5	6.49 d 2.4
C1''-H	1	5.08 d 7.3		5.10 d 7.5	5.08 d 7.6
sugar protons		3.1-3.6 (6 sugar protons)		3.30-3.80 m (6 sugar protons)	3.22-3.60 m, (C2''-H to C5''-H)
					3.61 br. d 9.2 (C6''-H_2)

Shift values found for the substance in fraction R10 agree well with the data given by Markham and Geiger (1994). Coupling constants given for C2'-H and C6'-H by Nawwar *et al.* (1989) and C5'-H by Pieroni *et al.* (1996) are obviously not correct. As shown for quercetin (No.13) and quercitrin (No.15) the proton at C6' of the B-ring couples with protons at C2'and C5', while the latter two protons only couple with one proton, respectively.

^{13}C-NMR-Parameters (100 MHz)

Assignment	δ [ppm] multiplicity J [Hz] R10 (DMSO-d_6)	Markham *et al.*, 1978 (DMSO-d_6)	Nawwar *et al.*, 1989 (DMSO-d_6)	Pieroni *et al.*, 1996 (DMSO-d_6)
C4	181.7	181.6	181.8	183.7
C2	164.3	164.5	164.5	166.7
C7	163.0	162.9	163.1	164.6
C5	161.0	161.1	161.1	162.7
C9	156.8	156.9	157.0	158.7
C4'	149.8	149.7	149.8	151.5
C3'	145.7	145.7	145.8	147.0
C1'	121.3	121.6	121.7	123.0
C6'	119.0	119.0	119.3	120.4
C5'	115.9	116.1	116.2	116.9

Assignment	δ [ppm] multiplicity J [Hz] R10 (DMSO-d_6)	Markham *et al.*, 1978 (DMSO-d_6)	Nawwar *et al.*, 1989 (DMSO-d_6)	Pieroni *et al.*, 1996 (DMSO-d_6)
C2’	113.5	113.7	113.9	114.0
C10	105.2	105.5	105.6	107.0
C3	103.0	103.2	103.5	103.3
C1”	99.8	100.4	100.3	101.5[a]
C6	99.4	99.7	99.8	101.0[a]
C8	94.6	94.9	95.0	96.3
C5”	77.1	77.3[a]	77.5	78.4[b]
C3”	76.3	76.6[a]	76.6	77.8[b]
C2”	73.0	73.3	73.3	74.3
C4”	69.4	70.0	70.0	70.8
C6”	60.5	61.0	61.0	62.4

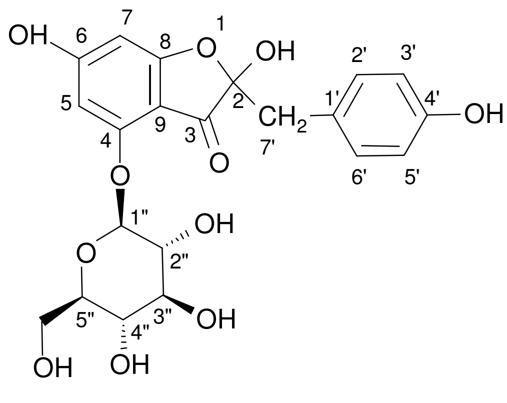
[a, b] Assignments bearing the same superscript in any one spectrum may be reversed.

Shift values of fraction R10 of the ^{13}C-NMR spectrum agree perfectly with data found in the literature. Hence it is concluded, that substance of R10 is luteolin-7-O-ß-D-glucopyranoside. A DEPT experiment was performed which corroborates the assignments depicted in the table.

11 Maesopsin-4-O-ß-D-glucopyranoside

Fractions	R2, (R8)	Formula	$C_{21}H_{22}O_{11}$
IUPAC-Name	2,4,6-Trihydroxy-2-[(4´-hydroxyphenyl) methyl]-3(2H)-benzofuranone-4-yl-ß-D-glucopyranoside	MW	450
INN-Names	maesopsin-4-O-ß-D-glucopyranoside		

Structure



TLC spot appearance (TLC, anisaldehyde / H_2SO_4): bright red-orange
hRf in TLC-system 3: 42-49

Characteristically, for maesopsin-glucoside: there was nearly no precipitation with lead acetate. Maesopsin-glucoside is a highly hydrophilic compound.

Maesopsin glucoside belongs to the group of aurones, in *sensu stricto* to the auronols, both forming a minor group of flavonoids. Numbering was followed as given by Mabry *et al.* (1970) and Bohm (1994).

HPLC

mean retention time	UV-Data λ_{max} / nm			
20.7 min. (R2)		sh 222	292	sh 345
21.9 min. (R8)		sh 223	291	sh 343
Yoshikawa K. et al., 1998b; (methanol)	210	228	280	

Fraction R2 and R8 show nearly the same UV spectra, but different retention times. Furthermore, as will be shown by mass spectrometry, fraction R8 has the same molecular weight and fragmentation pattern as compound of fraction R2. However, as there was too low substance of R8 to get a useful ^{1}H-NMR spectrum, it cannot be unambigously concluded at this point whether R8 is the same substance as R2 or an isomer, e.g. concerning the linkage of the glycoside (at C4 or C6) and its sugar moiety.

EI-MS (R2, very pure fraction)

m/z 288	(5 %)	$C_{15}H_{12}O_6$:	$[M]^{+\bullet}$, maesopsin (aglycone)
m/z 270	(14 %)	$C_{15}H_{10}O_5$:	$[M - H_2O]^{+\bullet}$
m/z 242	(3 %)	$C_{14}H_{10}O_4$:	$[M - H_2O - CO]^{+\bullet}$
m/z 181	(13 %)	$C_8H_5O_5$:	$[M - \text{4-hydroxybenzyl}]^+$, i.e. $[288\text{-}107]^+$
m/z 153	(24 %)	$C_7H_5O_4$:	$[A_1 + H]^+$
m/z 107	(100 %)	C_7H_7O:	4-hydroxybenzyl cation
m/z 73	(10 %)		
m/z 60	(16 %)		
m/z 51	(21 %)		

In accordance with Mabry and Markham (1975) the class of aurones showed a similar fragmentation pattern as did the flavones and flavonols (Fig. C.7, p. 102) under electron impact conditions. In accordance with an EI-MS spectrum on maesopsin aglycone (Li *et al.*, 1997), base peak of this spectrum is constituted by m/z 107, the p-hydroxybenzoyl moiety.
Again, the retro-Diels-Alder reaction is apparently a characteristic and predominantly occurring fragmentation pathway for this type of flavonoid.

To verify the atomic composition of the aglycone in this study, a high resolution measurement was performed.
High resolution mass spectrometry gave a precise molecular weight of 288.06292 amu which was found to correspond to a molecular composition of $C_{15}H_{12}O_6$, with 288.06339 amu calculated as theoretical mass. Hence, the ratio of observed to calculated mass amounts to 1.6 ppm.
The molecular weight of the corresponding auronol glycoside was evidenced by complementary information of the positive and negative ESI mode.

ESI-MS (R2)

positive mode:

m/z 489 (5 %)	:	$[M + K]^+$
m/z 473 (100 %)	:	$[M + Na]^+$
MS/MS of mass 473		
m/z 473 (31 %)	:	$[M + Na]^+$
m/z 455 (18 %)	:	$[M - H_2O + Na]^+$
m/z 437 (5 %)	:	$[M -2\ H_2O + Na]^+$
m/z 418 (2 %)	:	$[M -3\ H_2O + Na]^+$
m/z 311 (100 %)	:	$[\text{aglycone} + Na]^+$
MS^3 of mass 311		
m/z 311 (9 %)	:	$[\text{aglycone} + Na]^+$
m/z 293 (58 %)	:	$[\text{aglycone} - H_2O + Na]^+$
m/z 283 (43 %)	:	$[\text{aglycone} - CO + Na]^+$
m/z 175 (100 %)	:	$[A_1 + Na]^+$

negative mode:

m/z 449 (100 %)	:	$[M - H]^-$
MS/MS of mass 449		
m/z 449 (14 %)	:	$[M - H]^-$
m/z 431 (9 %)	:	$[M - H_2O - H]^-$
m/z 287 (100 %)	:	$[\text{aglycone} - H]^-$
m/z 269 (20 %)	:	$[\text{aglycone} - H_2O - H]^-$
m/z 259 (11 %)	:	$[\text{aglycone} - CO - H]^-$
MS^3 of mass 287		
m/z 287 (12.5 %)	:	$[\text{aglycone} - H]^-$
m/z 259 (100 %)	:	$[\text{aglycone} - H - CO]^-$

ESI-MS (R8)

positive mode:

m/z 473 (7 %)	:	$[M + Na]^+$
m/z 289 (32 %)	:	$[\text{maesopsin} + H]^+$, aglycone
m/z 191 (100 %)	:	unknown compound (like in fraction R7)

negative mode:

m/z 449 (12 %)	:	$[M - H]^-$
m/z 287 (100 %)	:	$[\text{maesopsin} - H]^-$, aglycone

The molecular weight of the auronol glycoside maesopsin-glucoside is conclusively determined as 450 Da.

NMR-Data

Maesopsin-4-glucoside exists as two diastereoisomers which is due to the attachment of a glucosyl moiety to the aglycone, which itself appears as pair of enantioisomers because of the hemiketal at C2, the chiral centre of the maesopsin aglycone. Therefore, a double set of signals was obtained in the ^1H- and ^{13}C-NMR spectra. As far as signals have been resolved, both sets of data are depicted in the respective tables of the ^1H-NMR and ^{13}C-NMR experiment. Assignments in the ^1H spectrum are based on ^1H and H,H-COSY experiments.
The ^{13}C NMR spectra were first measured in D_2O and afterwards in DMSO-d_6. In D_2O shift values were referenced to methanol (delta (H) = 3.247 ppm and delta (C) = 49.384 ppm).

^{1}H-NMR-Parameters

Assignment	No. of H	δ [ppm] multiplicity J [Hz] R2 (DMSO-d_6) Diast. (58%)	R2 (DMSO-d_6) Diast. (42%)	Yoshikawa *et al.*, 1998b (DMSO-d_6)	Yoshikawa *et al.*, 1998b (pyridine-d_5)
-OH		9.4 v.br. 7.77 br. 5.75 br. 5.22 br.		C6-OH 9.25 (1H) s, br. C4'-OH 7.61 (0.4 H) s, br. and 7.63 (0.6 H) s, br.	
C2'-H/ C6'-H	2	6.914 AA'XX', N = 8.5	6.919	6.93 (2H) d 8.5	7.54 d 8.5 and 7.55 d 8.5
C3'-H/ C5'-H	2	6.543 AA'XX', N = 8.5	6.536	6.55 (2H) d 8.5	7.02 d 8.5 and 7.08 d 8.5
C5-H	1	5.97 d 1.6[a]	5.99 d 1.6[b]	5.98 (0.6 H) 6.02 (0.4 H), each d 1.5	6.59 d 1.5 and 6.67 d 1.5
C7-H	1	5.94 d 1.6[a]	5.92 d 1.6[b]	5.92 d 1.5	6.41 d 1.5 and 6.47 d 1.5
C1"-H	1	4.85 m	4.91 m	4.88 (0.6 H) d 8.8 and 4.95 (0.4 H) d 8.0	5.50 d 8.0 and 5.60 d 8.0
C6"-H_2 (a) (b)	2	3.64 dd 11.8, 6.3 3.48 dd 11.8, 5.0			4.36 dd 11.0, 2.5; 4.27 dd 11.0, 5.5 and 4.36 dd 11.0, 2.5; 4.27 dd 11.0, 5.5
C2"-H	1	3.34-3.26 m			4.35 dd 8.0, 8.0 and 4.35 dd 8.0, 8.0
C3"-H	1				4.29 dd 8.0, 8.0 and 4.30 dd 8.0, 8.0
C5"-H	1			4.58 (0.6 H) m 4.67 (0.4 H) m	3.92 m and 3.92 m
C4"-H	1	3.21 m			4.22 dd 8.0, 8.0 and 4.28 dd 8.0, 8.0
C7'-H (a) (CH_2) (b)	2	2.987 d 13.90 2.921 d 13.90	2.960 d 13.95 2.903 d 13.95	2.89 and 2.96, each 1H and d 14.0	3.69 s (2H) and 3.62 d 14.0; 3.52 d 14.0

[a, b] Assignments bearing the same character may be reversed.

C2, C3, C4, C6, C8, C9, C1' and C4' are quaternary carbons and hence bearing no protons.

^{13}C-NMR-Parameters

Assignment	δ [ppm] multiplicity J [Hz] R2 (D_2O)	R2 (DMSO-d_6)	Yoshikawa *et al.*, 1998b (pyridine-d_5)
C3	196.3 and 196.4	192.5 and 192.9	195.50 and 195.54
C8	172.9 and 173.0	172.1	173.79 and 173.70
C6	170.1 and 170.2	168.6	170.95 and 170.89
C4	156.8 and 156.9	156.9	158.51 and 158.43
C4'	154.9 and 155.0	156.1	157.90 and 157.89

Assignment	δ [ppm] multiplicity J [Hz] R2 (D_2O)	R2 (DMSO-d_6)	Yoshikawa *et al.*, 1998b (pyridine-d_5)
C2’, C6’	132.16 and 132.17	131.2	132.70 and 132.70
C1’	125.01 and 125.02	124.3	125.65 and 125.57
C3’, C5’	115.39 and 115.41	114.5	115.95 and 115.87
C2	106.7 and 106.8	105.6	107.52 and 107.47
C9	102.45 and 102.54	101.9 and 102.2	103.25 and 103.25
C1”	99.7 and 99.9	99.1 and 99.3	102.05 and 101.72
C5	96.8 and 97.0	95.2 and 95.7	97.13 and 97.35
C7	93.3 and 93.4	91.4 and 91.6	93.21 and 93.28
C5”	76.62 and 76.64	77.0	79.15 and 79.02
C3”	75.6 and 75.7	76.5	78.24 and 78.34
C2”	72.61 and 72.61	72.8	74.26 and 74.39
C4”	69.66 and 69.70	69.1	70.99 and 70.94
C6”	60.88 and 60.93	60.2	62.22 and 62.22
C7’	40.7	40.4	41.90 and 42.08

To elucidate the structure of the auronol glycoside, in particular to identify unambigously the position bearing the glucosyl moiety (i.e. the attachment of the sugar moiety to the C4 or C6 hydroxyl of the aglycone), various two-dimensional experiments were performed: DQF-COSY, HSQC, HSQC-TOCSY and HMBC. The majority of information was revealed in the HMBC experiment, and showed a $^4J_{CH}$ coupling between the quaternary carbon atom C9 and the anomeric proton C1”-H of the glucose as depicted in Fig. II.4.

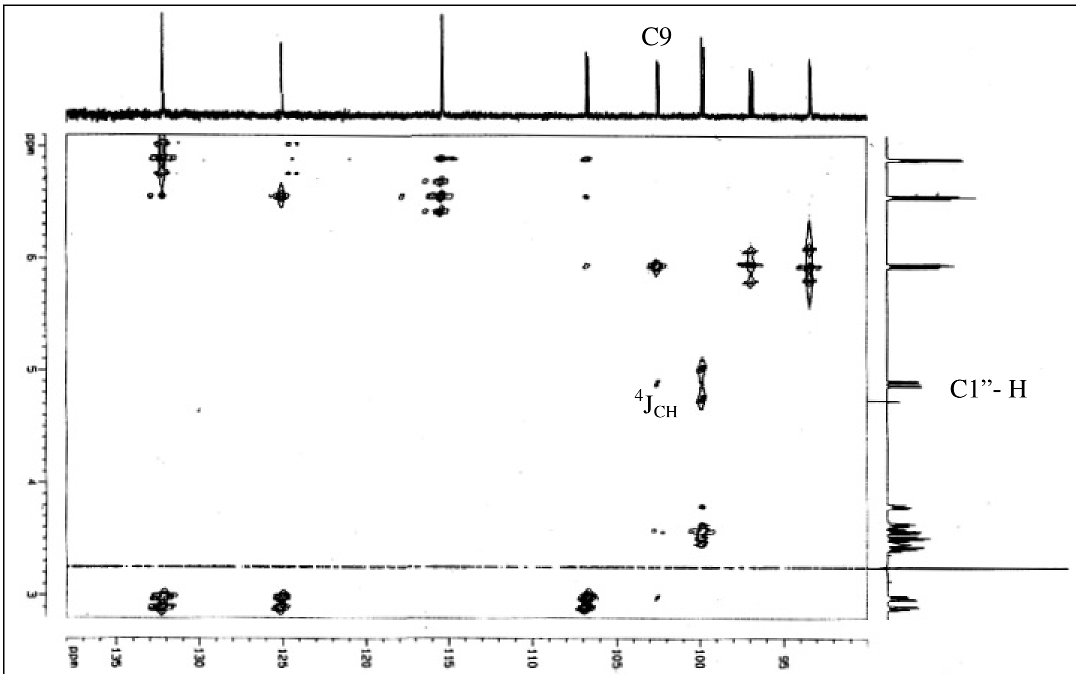
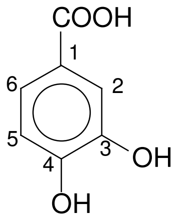


Fig. II.4: HMBC-spectrum.

12 Protocatechuic acid

Fractions	A9, R4, R5	Formula	$C_7H_6O_4$
IUPAC-Name	benzoic acid, 4,3-dihydroxy-	MW	154
INN-Names	protocatechuic acid	Cas. No.	[99-50-3]

Structure



HPLC

mean retention time	UV-Data / nm				
	λ_{max}	λ_{min}	λ_{max}	λ_{min}	λ_{max}
14.9 min.	217	235	259	279	294
Van Sumere, 1989 (MeOH)		235	258	278	292
Impurities: 15.6 min. (R4)	215		274		
16.2 min. (R4)	216		275		

EI-MS (average of R4 and R5)

m/z 154	(100 %)	$C_7H_6O_4$:		$[M]^{+\bullet}$
m/z 137	(73 %)	$C_7H_5O_3$:	- $OH^\bullet$	$[M - OH]^+$
m/z 109	(20 %)	$C_6H_5O_2$:		$[M - OH- CO]^+$, brenzcatechin cation
m/z 81	(8 %)	C_5H_5O:		$[M - OH- CO - CO]^+$
m/z 53	(16 %)	C_4H_5:		$[M - OH- CO - CO - CO]^+$

EI-MS (A9)

m/z 154	(84 %)	$C_7H_6O_4$:		$[M]^{+\bullet}$
m/z 137	(100 %)	$C_7H_5O_3$:	- $OH^\bullet$	$[M - OH]^+$
m/z 109	(46 %)	$C_6H_5O_2$:		$[M - OH- CO]^+$, brenzcatechin cation
m/z 81	(53 %)	C_5H_5O:		$[M - OH- CO - CO]^+$
m/z 63	(54 %)	C_5H_3:		$[M - OH- CO - CO - H_2O]^+$
m/z 55	(35 %)	C_3H_3O:		$[M - OH- CO - CO - C_2H_2]^+$
m/z 53	(26 %)	C_4H_5:		$[M - OH- CO - CO - CO]^+$

While fractions R4 and R5 showed the same fragmentation pattern (the average relative abundance of both fractions is given, respectively), fraction A9 showed three different fragments originating from m/z 81. In the latter case, base peak is m/z 137 instead of the molecule radical cation, m/z 154.

Characteristic ions given by Bocchi *et al.* (1996) for the EI experiment are largely in accordance with those detected in this study [m/z 154 (96 %); m/z 137 (100 %); m/z 109 (29 %)]. However, m/z 136 (6 %) for $[M - H_2O]^{+\bullet}$ could not be confirmed by the spectra of fractions studied here.

ESI-MS (A9)

negative mode:

m/z 153	(66 %)	:	$[M - H]^-$
m/z 109	(100 %)	:	$[\text{brenzcatechin - H}]^-$
impurity:			
m/z 169	(24 %)	:	$[\text{gallic acid - H}]^-$

^{1}H-NMR-Parameters

Assignment	No. of H	δ [ppm] multiplicity J [Hz] A9, R4, R5 (DMSO-d_6)	Draths and Frost, 1991 (D_2O)	Scott, 1970 (acetone-d_6)
C2-H	1	7.33 d 2.0	7.52 d 1.0	7.58 d 2.0
C6-H	1	7.28 dd 8.3, 2.0	7.55 dd[a] 7.1	7.52 dd 8.3, 2.0
C5-H	1	6.77 d 8.2	6.97 dd[b] 7.1	6.94 d 8.3

[a] The second coupling constant is not given in that reference.

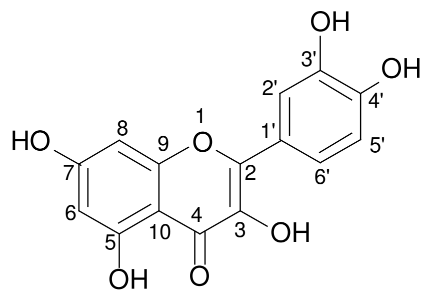
[b] Obvious error in the reference regarding the multiplicity.

Values given for the ^{1}H-NMR-spectrum are mean values of all three fractions.
There was insufficient protocatechuic acid isolated to perform a ^{13}C-NMR experiment (for a ^{13}C-NMR see Scott (1972, in acetone-d_6).

13 Quercetin

Fractions	A15, A16, A23, R18	Formula	$C_{15}H_{10}O_7$
IUPAC-Name	2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one	MW	302
INN-Names	quercetin	Cas. No.	[117-39-5]

Structure



Quercetin is the main component of fraction A15, with a side-compound is afzelin.
It can be presumed that quercetin is an artefact generated through analytical procedures, as no free aglyca could be found by other methods (see chapter C.III.3.1).

HPLC

mean retention time	UV-Data λ_{max} / nm			
32.5 min.	255	sh 269	sh 297	370
Mabry et al., 1970 (MeOH)	255	sh 269	sh 301	370
impurity: 29.4 min. (A15, afzelin, like A14)	263	sh 292	sh 315	343

UV-data given are average values of all three fractions.

Mass spectrometry

In the following, fractions A15 and A16 are documented as representative fractions.

EI-MS (data averaged on A15 and A16)

m/z 302 (100 %) $C_{15}H_{10}O_7$: $[M]^{+\bullet}$, quercetin
m/z 274 (4 %) $C_{14}H_{10}O_6$: $[M - CO]^{+\bullet}$
m/z 273 (4 %) $C_{14}H_9O_6$: $[M - CO - H]^+$
m/z 257 (2 %)
m/z 245 (4 %) $C_{13}H_9O_5$: $[M - CO - CO - H]^+$
m/z 229 (4 %)
m/z 153 (5 %) $C_7H_5O_4$: $[A_1 + H]^+$
m/z 142 (4 %)
m/z 137 (6 %) $C_7H_5O_3$: $[B_2]^+$
m/z 128 (6 %)
m/z 109 (3 %) $C_6H_5O_2$: $[B_2 - CO]^+$
m/z 69 (6 %)
impurity of A15 and A16:
m/z 286 (6 %) $C_{15}H_{10}O_6$: $[\text{kaempferol}]^{+\bullet}$

ESI-MS

positive mode (A16):
m/z 303 (80 %) : $[M + H]^+$
impurity:
m/z 471 (24 %) : [afzelin + K]$^+$, kaempferol glycoside
m/z 455 (100 %) : [afzelin + Na]$^+$, kaempferol glycoside
positive mode (A15 and A23):
m/z 303 (100 %) : $[M + H]^+$
impurity of A15:
m/z 455 (20 %) : [afzelin + Na]$^+$, kaempferol glycoside
m/z 287 (27 %) : [kaempferol + H]$^+$, aglycone
negative mode (all three fractions):
m/z 301 (100 %) : $[M - H]^-$

NMR-Data

Fraction A16 was used as a representative spectrum to document the ^{1}H experiment, while the ^{13}C experiment could only be performed with an authentic sample (quercetin purchased from Merck) as not enough substance was isolated. Commercial quercetin was also used to verify the ^{1}H-NMR spectrum and to perform two-dimensional spectra.

^{1}H-NMR-Parameters

The authentic sample was measured at 360 MHz.

Signal	Assign-ment	No. of H	δ [ppm] multiplicity J [Hz] A16 (DMSO-d_6)	authentic sample (quercetin, Merck; DMSO-d_6)	Barberá *et al.*, 1986 (DMSO-d_6)	Shen *et al.*, 1993 (DMSO-d_6)	Markham and Geiger, 1994 (DMSO-d_6)
A	C5-OH	1	12.5 s, br.	12.49	12.5-12.7 s, br.	12.42 s	12.45
B	C7-OH	1		10.76			-OH
C	C4'-OH	1		9.57			-OH
D	C3-OH	1		9.34			-OH
E	C3'-OH	1		9.28			-OH
F	C2'-H	1	7.67 d 2.2	7.67 d 2.3	7.67 d 2.2	7.67 d 2.2	7,69 d 2.1
G	C6'-H	1	7.54 dd 8.6, 2.2	7.54 dd 8.4, 2.3	7.54 dd 8.5, 2.2	7.53 dd 8.6, 2.2	7.55 dd 8.5, 2.1
H	C5'-H	1	6.88 d 8.6	6.88 d 8.4	6.90 d 8.5	6.89 d 8.3	6.90 d 8.5
I	C8-H	1	6.40 d 2.0	6.40 d 2.0	6.40 d 2.0	6.40 d 2.0	6.42 d 2.1
K	C6-H	1	6.18 d 2.0	6.14 d 2.0	6.20 d 2.0	6.18 d 2.0	6.20 d 2.1

The assignments of the hydroxyl groups at C3, C5, C7 of ring A and C, and C3' and C4'of ring B were found by two-dimensional NMR-spectroscopy and could be unambigously assigned in this study for the first time. Furthermore, C5' and C2' could be unambiguously assigned by ^{13}C spectroscopy.

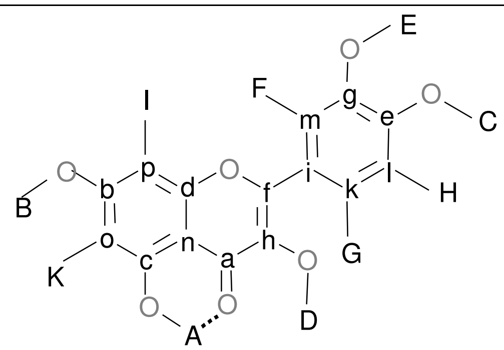


Fig. II.5: Characters (capitals indicate protons, small letters the carbon atoms) are used for assignments gained by two-dimensional experiments.

^{13}C-NMR-Parameters

The authentic sample was measured at 90 MHz.

Signal	Assignment	δ [ppm] multiplicity J [Hz] authentic sample (Merck) (DMSO-d$_6$)	Shen *et al.*, 1993 (DMSO-d$_6$)	Shigematsu *et al.*, 1982 (DMSO-d$_6$)	Nawwar *et al.*, 1984b (DMSO-d$_6$)	Markham *et al.*, 1978 (DMSO-d$_6$)
a	C4	175.7	175.7	175.8	175.8	175.8
b	C7	163.8	163.8	163.8	163.9	163.9
c	C5	160.6	160.6	160.6	160.7	160.7
d	C9	156.0	156.1	156.1	156.2	156.2
e	C4’	147.6	147.6	147.6	147.6	147.6
f	C2	146.7	146.8	146.7	146.9	146.9
g	C3’	145.0	145.0	145.0	145.0	145.0
h	C3	135.6	135.6	135.6	135.5	135.5
i	C1’	121.9	121.9	122.0	122.1	122.1
k	C6’	119.9	119.9	120.0	120.0	120.0
l	C5’	115.5	115.5	115.5	115.6	115.6[a]
m	C2’	115.0	115.1	115.0	115.3	115.3[a]
n	C10	102.9	103.0	102.9	103.1	103.1
o	C6	98.1	98.1	98.1	98.2	98.2
p	C8	93.2	93.3	93.3	93.3	93.3

[a] Shift values bearing the same superscript may be reversed.

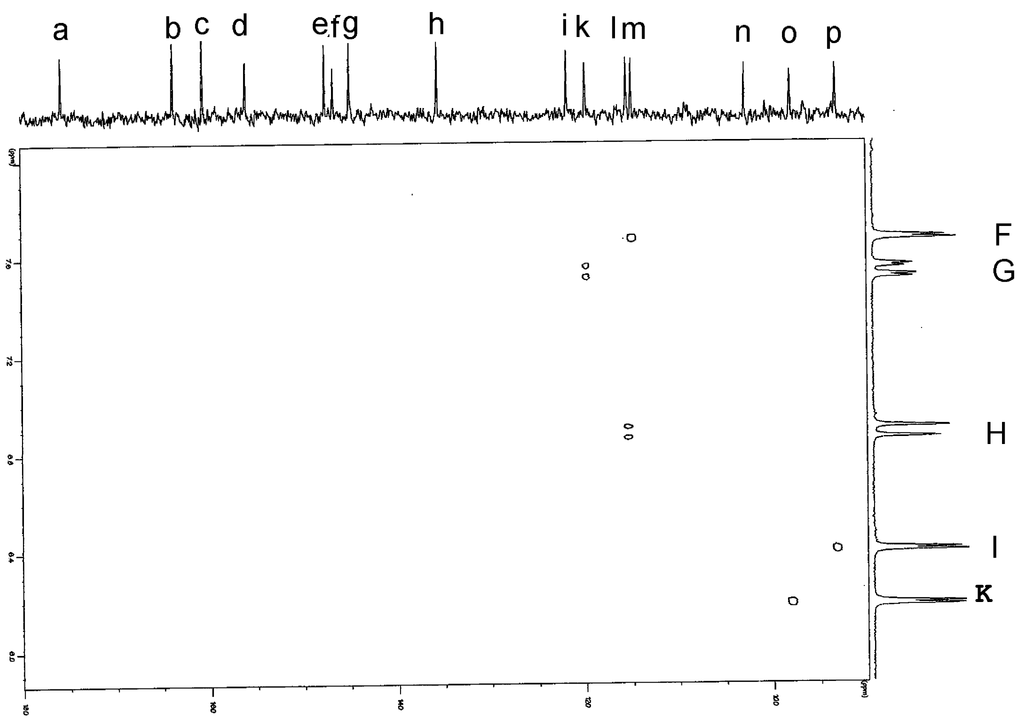


Fig. II.6: HSQC-spectrum of quercetin.

Couplings over a short (HSQC) and long distance (HMBC) between proton and carbon atoms are summarised in Tab. II.4.

Tab. II.4: H-C heterocorrelations of quercetin based on HSQC and HMBC experiments (DMSO-d_6, 360 MHz).

^{1}H / ^{13}C	C5-OH A	C7-OH B	C4'-OH C	C3-OH D	C3'-OH E	C2'-H F	C6'-H G	C5'-H H	C8-H I	C6-H K
C4 a	4;2[b]			3					4[c]	4[c]
C7 b	4[c]	2							2	2
C5 c	2									2
C9 d									2	
C4' e			2		3	3	3	2		
C2 f				3		3	3	4		
C3' g			3		2	2	4[d]	3		
C3 h				2						
C1' i						2	[e]	3		
C6' k						3	1	[e]		
C5' l			3				[e]	1		
C2' m					3	1	3	4[ad]		
C10 n	3								3	3
C6 o	3	3							3	1
C8 p		3							1	3

[a] weak correlation

[b] dual coupling pathway through intramolecular H-bond

[c] w-coupling

[d] dual coupling pathway

[e] missing two-bond correlation

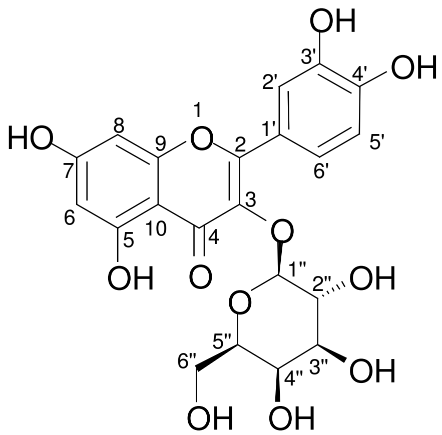
Correlations crucial to the OH assignments are shaded.

C-H shift correlations (Tab. II.4) across one bond (HSQC, Fig. II.6) or more bonds (HMBC, the spectrum is not depicted here) are in perfect agreement with the assignments reported in the literature and in addition, yield the hitherto unknown assignments of the hydroxyl protons B to E. All expected vicinal correlations are observed. Of the 15 possible geminal correlations, which are usually weaker than vicinal ones, three were not detected. The seven visible, four-bond correlations are either transmitted across dual coupling pathways or fixed w-configurations. Each hydroxyl proton shows at least one correlation that allows an unequivocal assignment.

14 Quercetin-3-O-ß-D-galactopyranoside = Hyperoside

Fractions	A11, A19	Formula	$C_{21}H_{20}O_{12}$
INN-Names	quercetin-3-O-ß-D-galactopyranoside, hyperoside, hyperin	MW	464
		Cas. No.	[482-36-0]

Structure



Appearance Yellow needles (Yasukawa and Takido, 1987).

HPLC

mean retention time	UV-Data λ max / nm			
25.4 min.	255	sh 266	sh 293	354
Yasukawa and Takido, 1987 (EtOH)	258	sh 270	sh 293	364
Shigematsu *et al.*, 1982 (EtOH)	260			367
Mabry *et al.*, 1970 (MeOH)	257	sh 269	sh 299	362
Kaneta *et al.*, 1980 (MeOH)	257	sh 270	sh 300	360
impurity: 26.3 min. (A19, quercetin glycoside)	255	sh 266	sh 293	355

EI-MS (A11)

m/z 302	(100 %)	$C_{15}H_{10}O_7$:	$[M]^{+\bullet}$, quercetin (aglycone)
m/z 273	(4 %)	$C_{14}H_9O_6$:	$[M - CO - H]^+$
m/z 257	(2 %)		
m/z 245	(3 %)	$C_{13}H_9O_5$:	$[M - CO - CO - H]^+$
m/z 229	(3 %)		
m/z 153	(5 %)	$C_7H_5O_4$:	$[A_1 + H]^+$
m/z 137	(7 %)	$C_7H_5O_3$:	$[B_2]^+$
m/z 73	(10 %)		

ESI-MS (A11)

positive mode:

m/z 503	(10 %)	:	$[M + K]^+$, quercetin glycoside
m/z 487	(69 %)	:	$[M + Na]^+$
m/z 465	(7 %)	:	$[M + H]^+$
m/z 303	(100 %)	:	$[aglycone + H]^+$, quercetin

negative mode:

m/z 463	(100 %)	:	$[M - H]^-$, quercetin glycoside
m/z 301	(9 %)	:	$[aglycone - H]^-$, quercetin

EI-MS (A19)

m/z 302	(100 %)	$C_{15}H_{10}O_7$:	$[M]^{+\bullet}$, quercetin (aglycone)
m/z 285	(3 %)	$C_{15}H_9O_6$:	$[M - OH]^+$
m/z 245	(4 %)	$C_{13}H_9O_5$:	$[M - CO - CO - H]^+$
m/z 144	(13 %)		
m/z 114	(10 %)		
m/z 97	(21 %)		
m/z 73	(55 %)		
m/z 69	(30 %)		
m/z 60	(71 %)		

ESI-MS (A19)

positive mode:

m/z 503	(13 %)	:	$[M + K]^+$, quercetin glycoside
m/z 487	(100 %)	:	$[M + Na]^+$
m/z 303	(20 %)	:	$[aglycone + H]^+$, quercetin

negative mode:

m/z 463	(100 %)	:	$[M - H]^-$, quercetin glycoside
m/z 301	(46 %)	:	$[aglycone - H]^-$, quercetin

Ref.: EI-MS: Kaneta *et al.*, 1980.

NMR-Data

Fraction A11 was used as representative sample.

^{1}H-NMR-Parameters

Assignment	No. of H	δ [ppm] multiplicity J [Hz] A11 (DMSO-d_6)	Yasukawa and Takido, 1987 (DMSO-d_6)	Bennini *et al.*, 1992 (DMSO-d_6)	Barberá *et al.*, 1986 (DMSO-d_6)	Markham and Geiger, 1994 (DMSO-d_6)
C5-OH	1	12.63 s, br.	12.65, s	12.60		12.61
C6'-H	1	7.67 dd 8.5, 2.1	7.60 dd 8.3, 2.0	7.65 dd 8.3, 2.2	7.66 dd 8.5, 2.2	7.68 dd 8.5, 2.0
C2'-H	1	7.54 d 2.1	7.59 d 2.0	7.52 d 2.2	7.55 d 2.2	7.55 d 2.0
C5'-H	1	6.82 d 8.5	6.86 d 8.3	6.80 d 8.3	6.83 d 8.5	6.84 d 8.5
C8-H	1	6.41 ca. d 2[a]	6.42 d 1.9	6.38 d 1.9	6.41 d 2.0	6.42 d 1.9
C6-H	1	6.20 ca. d 2[a]	6.21 d 1.9	6.18 d 1.9	6.21 d 2.0	6.22 d 1.9
C1''-H	1	5.38 d 7.7	5.52 d 7.3	5.35 d 7.6	5.36 d 7.6	
C2''-H	1	3.58 ca. t 8.6[a]		3.56 dd 9.4, 7.6		
C3''-H	1 of 3	3.4-3.3		3.36 dd 9.4, 3.2		

Assignment	No. of H	δ [ppm] multiplicity J [Hz] A11 (DMSO-d_6)	Yasukawa and Takido, 1987 (DMSO-d_6)	Bennini *et al.*, 1992 (DMSO-d_6)	Barberá *et al.*, 1986 (DMSO-d_6)	Markham and Geiger, 1994 (DMSO-d_6)
C4”-H	1	3.66 d 3.0		3.65, b d 3.2		
C5”-H	1 of 3	3.4-3.3		3.33, m		
C6”-Ha	1 of 3	3.4-3.3				
C6”-Hb	1	3.46 dd 9.5, 5.1		3.33, m and 3.46 dd 9.5, 5.1		

[a] signals were not completely resolved

As protons at the C6” of the galactose moiety have a different chemical environment due to the chiral centre at C5”, depending on the degree of resolution two signals can be expected. Hence, two signals could be ascertained in this study and were reported by Bennini *et al.* (1992).

^{13}C-NMR-Parameters

Assign ment	δ [ppm] multiplicity J [Hz] A11 (DMSO-d_6)	Shigematsu *et al.*, 1982 (DMSO-d_6)	Yasukawa and Takido, 1987 (DMSO-d_6)	Markham *et al.*, 1978 (DMSO-d_6)	Bennini *et al.*, 1992 (DMSO-d_6)	Barberá *et al.*, 1986 (DMSO-d_6)
C4	177.5	177.4	177.6	177.5	177.4	177.37
C7	164.3	164.0	164.2	164.0	164.2	164.05
C5	161.2	161.1	161.3	161.2	161.1	161.10
C9[a]	156.3	156.3	156.5, (C2/C9)	156.3	156.2	156.20[a]
C2[a]	156.2	156.3	156.5, (C2/C9)	156.3	156.2	156.18[a]
C4’	148.5	148.4	148.4	148.3	148.4	148.31
C3’	144.9	144.7	144.8	144.7	144.7	144.66
C3	133.5	133.4	133.8	133.8	133.4	133.49
C6’	122.0	121.9; C1´	121.9	121.8	121.9	121.79
C1’	121.1	121.0; C6´	121.4	121.3	121.0	121.05
C2’	116.0	115.9; C5´	116.4; C5´	116.2, C5´ [a]	115.1, C2´	115.08, C2´
C5’	115.2	115.1; C2´	115.3; C2´	115.2, C2´ [a]	115.9, C5´	115.94, C5´
C10	103.9	103.9	104.2	104.0	103.8	103.82
C1”	101.8	101.9	102.6	102.3	101.8	101.93
C6	98.7	98.6	98.8	98.6	98.6	98.56
C8	93.5	93.5	93.6	93.4	93.4	93.38
C5”	75.9	75.7	75.9	75.8	75.7	75.69
C3”	73.2	73.2	73.6	73.4	73.2	73.17
C2”	71.2	71.2	71.5	71.3	71.2	71.15
C4”	67.9	67.9	68.2	68.0	67.9	67.84
C6”	60.1	60.1	60.4	60.8	60.1	60.04

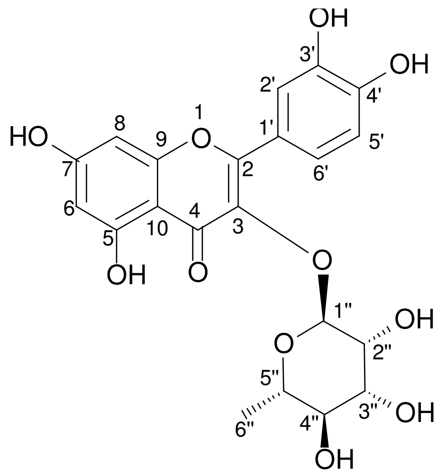
[a] Assignments bearing the same superscripts in either column may be reversed.

The assignments for C1´and C6´ in the spectrum of Shigematsu *et al.* (1982) should be interchanged, because the DEPT-spectrum of that substance only showed a signal at 121.995 ppm, indicating that there must be a primary or tertiary carbon atom, which is true for the C6´ as tertiary carbon atom. The C1´carbon is a quaternary carbon atom and therefore not visible in the DEPT-spectrum. These findings are in accordance with the assignments of other cited authors.

15 Quercetin-3-O-α-L-rhamnopyranoside = Quercitrin

Fractions	A12, R11 For evaluation of fraction R11 see above (see for luteolin-7-O-glucopyranoside).	Formula	$C_{21}H_{20}O_{11}$
IUPAC-Name	4H-1-benzopyran-4-one, 3-[(6-deoxy-α-L-mannopyranosyl) oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy	MW	448
INN-Names	quercetin-3-O-α-L-rhamnopyranoside, quercitrin	Cas. No.	[522-12-3]

Structure



Appearance Pale yellowish powder (Fukunaga *et al.*, 1988) or yellow needles (Tanaka *et al.*, 1984)

Commercial quercitrin (Roth) was used as authentic sample.

HPLC

mean retention time	UV-Data λ max / nm			
27.2 min.	255	sh 265	sh 298	349
Mabry *et al.*, 1970 (MeOH)	256	sh 265	sh 301	350
Fukunaga *et al.*, 1988 (EtOH)	255	sh 265		349
Tanaka *et al.*, 1984 (MeOH)	257	sh 264		350
Curir *et al.*, 1996 (MeOH)	256.0	sh 264.9	sh 301	349.8
Kaneta *et al.*, 1980 (MeOH)	256	sh 265	sh 300	350
Impurities: 26.6 min. (A12, a kaempferol derivative) For further compounds in R11 see luteolin-7-O-glucopyranoside)	265	sh 293	sh 318 (weak)	347

Mass spectrometry

Fraction A12 was used to document quercitrin representatively.

EI-MS

m/z 302	(100 %)	$C_{15}H_{10}O_7$:	$[M]^{+\bullet}$, quercetin aglycone
m/z 274	(4 %)	$C_{14}H_{10}O_6$:	$[M - CO]^{+\bullet}$
m/z 257	(2 %)	$C_{14}H_9O_5$:	$[M - CO - OH]^+$
m/z 245	(3 %)	$C_{13}H_9O_5$:	$[M - CO - CO - H]^+$
m/z 229	(4 %)	$C_{13}H_9O_4$:	$[M - CO - CO - OH]^+$
m/z 153	(6 %)	$C_7H_5O_4$:	$[A_1 + H]^+$, for both aglyca, quercetin and the impurity kaempferol
m/z 137	(7 %)	$C_7H_5O_3$:	$[B_2]^+$
m/z 113			
impurity:			
m/z 286	(4 %)	$C_{15}H_{10}O_6$:	$[kaempferol]^{+\bullet}$, aglycone

ESI-MS

positive mode:

m/z 487	(17 %)	:	$[M + K]^+$, quercitrin
m/z 471	(44 %)	:	$[M + Na]^+$
m/z 449	(5 %)	:	$[M + H]^+$
m/z 303	(100 %)	:	$[aglycone + H]^+$, quercetin
impurity:			
m/z 287	(21 %)	:	$[aglycone + H]^+$, kaempferol

negative mode:

m/z 447	(100 %)	:	$[M - H]^-$, quercitrin
m/z 301	(7 %)	:	$[aglycone - H]^-$, quercetin

For details of fraction R11 see luteolin-7-O-ß-D-glucopyranoside.
Ref.: EI-MS: Kaneta *et al.* (1980).

^{1}H-NMR-Parameters

Signal	Assig-ment	No. of H	δ [ppm] multiplicity J [Hz] A12 (DMSO-d_6)	authentic sample, (CD_3OD)	Curir *et al.*, 1996 (DMSO-d_6)	Markham *et al.*, 1992 (DMSO-d_6)	Özipek *et al.*, 1994 (CD_3OD)	Slowing *et al.*, 1994 (CD_3OD)
A	C5-OH	1	12.63 s					
B	C7-OH	1	10.88					
C	C4'-OH	1	9.72					
D	C3'-OH	1	9.36					
E	C2'-H	1	7.30 d 2.2	7.34 d 2.2	7.35 d 2		7.33 d 2.1	7.41 d 2.1
F	C6'-H	1	7.26 dd 8.3, 2.2	7.31 dd 8.3, 2.2	7.29 dd 8, 2		7.29 dd 8.4, 2.1	7.39 dd 8.1, 2.1
G	C5'-H	1	6.87 d 8.3	6.91 d 8.3	6.92 d 8		6.90 d 8.4	7.00 d 8.1
H	C8-H	1	6.39 d 1.9	6.37 d 2.1	6.41 AB 2.5		6.36 d 2.1	6.45 d 2.1
I	C6-H	1	6.21 d 1.9	6.20 d 2.1	6.24 AB 2.5		6.19 d 2.1	6.28 d 2.1
K	C1"-H	1	5.26 d 1.4	5.35 d 1.6	5.31 br.	5.25 d 1.1	5.34 d 1.6	5.43 d 1.5
L	C2"-OH	1	4.94 br.					
M	C4"-OH	1	4.71 br.					
N	C3"-OH	1	4.63 br.					
O	C2"-H	1	3.98 dd 3.1, 1.4	4.22 dd 3.4, 1.7	4.03 br. d 4.5	4.00 br. d 3.1	4.21 dd 3.4, 1.6	4.30 dd 3.3, 1.5
P	C3"-H	1	3.51 dd 8.7 ± 0.4, 3.1	3.75 dd 9.4, 3.4	3.58 dd 9, 4.5	3.53 dd 8.8, 3.1	3.74 dd 9.3, 3.4	3.82 dd 9, 3.3
Q	C5"-H	1	3.22 <u>A</u>Bq 9.4, 6.0	3.43 dq 9.7, 6.0	3.34 dq 9, 6	3.24 dq 9.5, 6	3.41 dq 9.3, 6.1	3.50 m
R	C4"-H	1	3.15 A<u>B</u>d 9.4, 8.7 ± 0.4	3.33 t 9.1	3.21 t 9	3.18 dd 9.3, 9	3.32 t 9.3	3.45 m
S	C6"-H_3	3	0.82 d 6.0	0.94 d 6.1	0.89 d 6	0.85 d 5.8	0.93 d 6.1	1.03 d 5.9

The hydroxyl groups of the aglycone are assigned by comparison to quercetin (see there), where signals could be unequivocally assigned by 2D-NMR experiments (^{1}H,^{1}H-COSY, HSQC, HMBC). The sugar -OH signals are assigned by vicinal correlations in the ^{1}H,^{1}H-COSY. These assignments have not been reported before. The other ^{1}H-data agree well with literature.

^{13}C-NMR-Parameters

Signal	Assign-ment	δ [ppm] multiplicity J [Hz] A12 (DMSO-d_6)	authentic sample, (CD_3OD)	Markham *et al.*, 1978 and Markham, 1989 (DMSO-d_6)	Markham *et al.*, 1992 (DMSO-d_6)	Slowing *et al.*, 1994 (CD_3OD)
a	C4	177.8	179.7	177.7		179.7
b	C7	164.3	165.9	164.0		165.9
c	C5	161.3	163.2	161.2		159.3[a]
d	C2	157.3	159.3[a]	156.4[a]		158.5[a]
e	C9	156.5	158.5[a]	157.0[a]		163.2
f	C4’	148.5	149.8	148.3		149.8
g	C3’	145.2	146.4	145.1		146.4
h	C3	134.2	136.3	134.4		136.2
i	C6’	121.1	123.0	121.0		122.8[d]
k	C1’	120.8	122.9	121.0		122.9[d]
l	C2’	115.7	116.9[b]	115.4[b]		116.9
m	C5’	115.5	116.5[b]	115.8[b]		116.3
n	C10	104.1	105.9	104.2		105.9
o	C1”	101.8	103.6	101.9	101.9	103.5
p	C6	98.7	99.8	98.6		99.8
q	C8	93.6	94.7	93.5		94.7
r	C4”	71.2	73.3	71.5	71.5	73.2
s	C5”	70.6	72.1[c]	70.1[c]	70.6	72.0
t	C3”	70.4	72.0[c]	70.6[c]	70.4	72.1
u	C2”	70.1	71.9[c]	70.4[c]	70.1	71.9
v	C6”	17.5	17.7	17.3	17.3	17.7

a - c Within one spectrum assignments bearing the same character may be reversed.

d Assignment obviously not correct as revealed by the DEPT experiment of this study.

The signal assignments reported in the literature are in part contradictory including a two-dimensional analysis given by Slowing *et al.* (1994) with an obviously incorrect assignment for C1’ and C6’ as revealed by DEPT in this study. Hence, in this study HSQC and HMBC heterocorrelations were performed allowing an unequivocal assignment of all carbon signals in the solvent DMSO-d_6.
The signal assignments in methanol generally follow the sequence found in DMSO, yet similar shift entries might be interchanged, except for C1’/ C6’, which is unequivocally based on DEPT.

Tab. II.5: H-C heterocorrelations for quercitrin based on HSQC and HMBC experiments (DMSO-d_6, 360 MHz).

^{1}H / ^{13}C		**C5-OH A**	**C2’-H E**	**C6’-H F**	**C5’-H G**	**C8-H H**	**C6-H I**	**C1”-H K**	**C2”-H O**	**C3”-H P**	**C5”-H Q**	**C4”-H R**	**C6”-H_3 S**
C4	**a**	4;2[b]				4[ac]	4[ac]						
C7	**b**	4[c]				2	2						
C5	**c**	2					2						
C2	**d**		3	3	4[ac]								
C9	**e**					2							

^{1}H / ^{13}C	C5-OH A	C2’-H E	C6’-H F	C5’-H G	C8-H H	C6-H I	C1”-H K	C2”-H O	C3”-H P	C5”-H Q	C4”-H R	C6”-H_3 S
C4’ f		3	3	2								
C3’ g		2		3								
C3 h							3					
C6’ i		3	1	2?								
C1’ k		2?	g	3								
C2’ l		1	3	4[d]?								
C5’ m		4[d]?	2	1								
C10 n	3				3	3						
C1” o							1	[e]	[f]			
C6 p	3				3	1						
C8 q					1	3						
C4” r								3	2	2	1	3
C5” s							3		[f]	1	2	2
C3” t							3?	2	1	3?	[e]	
C2” u							[e]	1	[e]		3[a]	
C6” v												1

[a] weak correlation

[b] dual coupling pathway through intramolecular H-bond

[c] w-coupling

[d] dual coupling pathway

[e] missing two-bond correlation

[f] missing three-bond correlation

For statistical reasons, in Tab. II.6 shift differences between quercetin and quercitrin are listed.

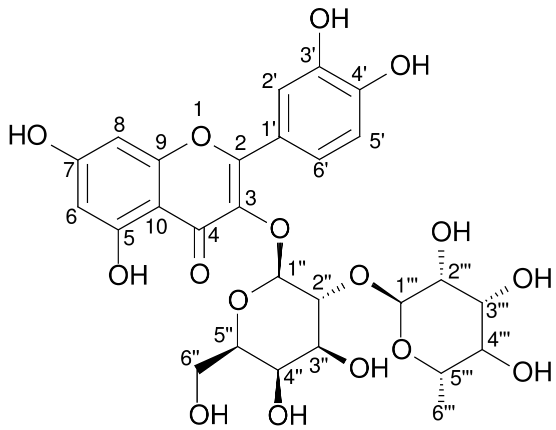
Tab. II.6: Shift differences between quercetin and quercitrin.

carbon	quercitrin	quercetin	quercitrin - quercetin
C2	157.297	146.709	10.588
C3	134.231	135.629	-1.398
C4	177.755	175.736	2.019
C5	161.314	160.620	0.694
C6	98.724	98.075	0.649
C7	164.297	163.780	0.517
C8	93.648	93.246	0.402
C9	156.470	156.031	0.439
C10	104.069	102.919	1.150
C1’	120.752	121.853	-1.101
C2’	115.671	114.973	0.698
C3’	145.224	144.958	0.266
C4’	148.461	147.600	0.861
C5’	115.477	115.504	-0.027
C6’	121.122	119.869	1.253

16 Quercetin-3-O-α-L-rhamnopyranosyl(1→2)ß-D-galactopyranoside

Fractions	A2	Formula	$C_{27}H_{30}O_{16}$
INN-Names	quercetin-3-O-α-L-rhamnopyranosyl (1→2)ß-D-galactopyranoside	MW	610

Structure



HPLC

mean retention time	UV-Data λ max / nm			
24.2 min.	255	sh 267	sh 294	355
Yasukawa and Takido, 1988 (EtOH)	258	sh 266		364

EI-MS

m/z 302 (100 %) $C_{15}H_{10}O_7$: $[M]^{+\bullet}$, quercetin (aglycone)
m/z 285 (2 %) $C_{15}H_9O_6$: - $OH^\bullet$ → $[M - OH]^+$
m/z 274 (5 %) $C_{14}H_{10}O_6$: $[M - CO]^{+\bullet}$
m/z 257 (2 %) $C_{14}H_9O_5$: $[M - CO - OH]^+$
m/z 245 (4 %) $C_{13}H_9O_5$: $[M - CO - CO - H]^+$
m/z 228 (5 %) $C_{13}H_8O_4$: $[M - CO - CO - H - OH]^+$
m/z 153 (6 %) $C_7H_5O_4$: $[A_1 + H]^+$
m/z 128 (12 %)
m/z 85 (14 %)
m/z 73 (17 %)

ESI-MS

positive mode:
m/z 649 (15 %) : $[M + K]^+$, quercetin glycoside
m/z 633 (100 %) : $[M + Na]^+$, quercetin glycoside
m/z 303 (22 %) : $[aglycone + H]^+$, quercetin
negative mode:
m/z 609 (100 %) : $[M - H]^-$, quercetin glycoside
m/z 301 (17 %) : $[aglycone - H]^-$, quercetin

^{1}H-NMR-Parameters

Assignment	No. of H	δ [ppm] multiplicity J [Hz] A2 (DMSO-d_6)	Yasukawa and Takido, 1988 (DMSO-d_6)
C5-OH	1	12.70 s	
C6'-H	1	7.72 dd 8.5, 2.2	7.65 dd 8.8, 1.9
C2'-H	1	7.48 d 2.2	7.52 d 1.9
C5'-H	1	6.80 d 8.5	6.82 d 8.8

Assignment	No. of H	δ [ppm] multiplicity J [Hz] A2 (DMSO-d_6)	Yasukawa and Takido, 1988 (DMSO-d_6)
C8-H	1	6.39 d 1.9	6.36 d 1.9
C6-H	1	6.19 d 1.9	6.18 d 1.9
C1”-H (gal)	1	5.63 d 7.7	5.61 d 7.3
C1”’-H (rha)	1	5.06 ca. d 2[a]	5.09 s
OH	1	5.02 s, br.	
not assigned	1	3.80 dd 9.4, 7.9	
sugar protons		3.77-3.22 m	
C4”’-H	1	3.12 t 9.5	
C6”’-H_3	3	0.77 d 6.2	0.86 d 6.3

[a] signals were not completely resolved

^{13}C-NMR-Parameters

Assignment	δ [ppm] multiplicity J [Hz] A2 (DMSO-d_6)	Yasukawa and Takido, 1988 (DMSO-d_6)
C4	177.3	177.3
C7	164.1	163.9
C5	161.3	161.3
C9	156.2	156.2
C2	156.0	156.3
C4’	148.4	148.2
C3’	144.9	144.7
C3	133.0	133.1
C6’	122.2	121.5
C1’	121.1	122.0
C5’	115.6	116.0
C2’	115.2	115.3
C10	103.9	104.2
C1”’	100.5	100.6
C1”	98.8	99.1
C6	98.7	98.7
C8	93.4	93.5
C5”	75.7	75.8
C2”	75.0	75.6
C3”	74.1	74.0
C4”’	71.9	72.3
C2”’	70.7	70.9
C3”’	70.6	70.7
C5”’	68.6	68.7
C4”	68.2	68.1
C6”	60.2	60.4
C6”’	17.2	17.1

Identification of compound A2 as quercetin-3-O-α-L-rhamnopyranosyl(1→2)ß-D-galactopyranoside is predominantly based on its ^{13}C-NMR spectrum, which perfectly agrees with data given by Yasukawa and Takido (1988). Shift values of relevant subspectra of analogue compounds (not depicted in this table) were used to corroborate the structure: kaempferol-3-O-α-L-rhamnopyranosyl(1→2)ß-D-galactopyranoside (compound No.9); quercetin-3-O-ß-D-apiofuranosyl-(1→2)ß-D-galactopyranoside (Hamburger *et al.*, 1985) and quercetin itself (compound No.13). (For ^{13}C shift values of pyranoses being in the free state or bound with respect to α- and ß-configurations, in D_2O and DMSO-d_6, see also Bailey and Butterfield (1981).) However, data from the ^{13}C-NMR experiment were decisive in determining the compound under investigation.

17 Quercetin-3-O-α-L-rhamnopyranosyl(1→2)ß-D-xylopyranoside

A novel substance

Fraction A5

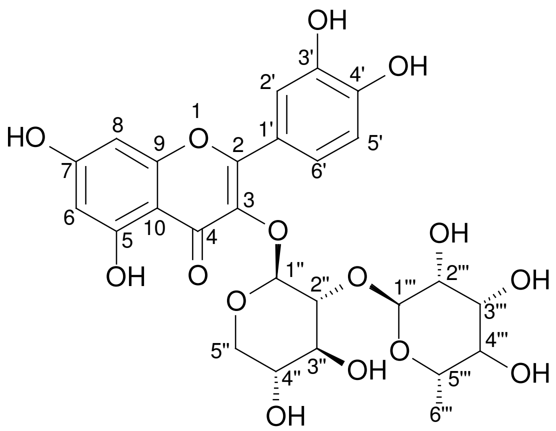
Formula $C_{26}H_{28}O_{15}$

IUPAC-Name

MW 580

INN-Names quercetin-3-O-α-L-rhamnopyranosyl (1→2)ß-D-xylopyranoside

Structure



HPLC

mean retention time	UV-Data λ max / nm			
25.8 min.	255	sh 267	sh 294	355

EI-MS

m/z 302	(100 %)	$C_{15}H_{10}O_7$:	$[M]^{+\bullet}$, quercetin (aglycone)
m/z 273	(5 %)	$C_{14}H_9O_6$:	$[M - CO - H]^+$
m/z 257	(2 %)	$C_{14}H_9O_5$:	$[M - CO - OH]^+$
m/z 245	(4 %)	$C_{13}H_9O_5$:	$[M - CO - CO - H]^+$
m/z 229	(4 %)	$C_{13}H_9O_4$:	$[M - CO - CO - OH]^+$
m/z 153	(6 %)	$C_7H_5O_4$:	$[A_1 + H]^+$
m/z 142	(5 %)		
m/z 137	(9 %)	$C_7H_5O_3$:	$[B_2]^+$
m/z 128	(13 %)		
m/z 114	(14 %)		
m/z 85	(15 %)		
m/z 73	(13 %)		
impurity:			
m/z 286		:	kaempferol aglycone (originating from a preceding fraction)

ESI-MS

positive mode:

m/z 603	(40 %)	:	$[M + Na]^+$, quercetin glycoside
m/z 303	(100 %)	:	$[aglycone + H]^+$, quercetin

negative mode:

m/z 579	(35 %)	:	$[M - H]^-$, quercetin glycoside
m/z 301	(100 %)	:	$[aglycone - H]^-$, quercetin

NMR-Data

For the novel compound A5, the assignment of the signals in the ^{1}H- and ^{13}C-spectra to their corresponding protons and carbon atoms is largely based on various two-dimensional experiments. To solve sophisticated problems such as to the type of binding of the anomeric sugar protons, suitable reference studies were used for the respective initial and terminal sugar moieties.

^{1}H-NMR-Parameters

Signal	Assignment	No. of H	δ [ppm] multiplicity J [Hz] A5 (DMSO-d_6)
A	C6'-H	1	7.57 dd 8.4, 2.2
B	C2'-H	1	7.54 d 2.2
C	C5'-H	1	6.85 d 8.4
D	C8-H	1	6.41 d 2.0
E	C6-H	1	6.19 d 2.0
F	C1''-H (xyl)	1	5.51 d 7.4
G	C1'''-H (rha)	1	5.08 d 1.4
H	C5'''-H	1	3.78 dq 9.4, 6.2
I	C2'''-H	1	3.74 dd 3.2, 1.4
J	C5''-H	1	3.60 m
K	C2''-H	1	3.55 dd 9.0, 7.4
L	C3'''-H	1	3.48 m[a]
M, N	C3''-H, C4''-H	2	3.34 m[a]
O	C4'''-H	1	3.15 t 9.5
P	C5''-H	1	2.96 m
Q	C6'''-H_3	3	0.88 d 6.2
R	C5-OH	1	12.62 s, br.
	-OH	0.6	9.3 v.br.
	-OH	ca. 1	5.33 br.
	-OH	ca. 1	5.00 br.
	-OH	ca. 1	4.63 br.
	-OH	ca. 1	4.58 br.
	-OH	ca. 1	4.50 br.

[a] signals were covered by a large peak of water

<u>Coupling constants J (Hz) determined for the coupling protons of the rhamnose moiety:</u>
J (C1'''-H / C2'''-H) = 1.4 Hz ; J (C2'''-H / C3'''-H) = 3.2 Hz
J (C3'''-H / C4'''-H) = 9.6 Hz ; J (C4'''-H / C5'''-H) = 9.4 Hz
J (C5'''-H / C6'''-H_3) = 6.2 Hz
<u>Coupling constants J (Hz) determined for the coupling protons of the xylose moiety:</u>
J (C1''-H / C2''-H) = 7.4 Hz ; J (C2''-H / C3''-H, C4''-H) = 9.0 Hz

The two-dimensional $^{1}H,^{1}H$-COSY experiment revealed the coupling protons and corroborated the assignment of the signals found in the ^{1}H-spectrum.

$^{1}H,^{1}H$-COSY		
proton	**coupling with**	
A	B, C	quercetin
D	E	
F	K	xylose
K	M, N	
M, N	P	
P	J	
Q	H	rhamnose
H	O	
O	L	
L	I	
I	G	

^{13}C-NMR-Parameters and data of two-dimensional experiments

Signal	Assignment	δ [ppm] multiplicity J [Hz] A5 (DMSO-d6)	HSQC with	HMBC with
a	C4	177.2	-	-
b	C7	164.2	-	D E
c	C5	161.2	-	E (R)
d	C9	156.2	-	D
e	C2	156.1	-	A B
f	C4’	148.6	-	A B (C)
g	C3’	145.0	-	B C A?
h	C3	132.8	-	(F)
i	C6’	121.5	A	B
j	C1’	121.0	-	C
k	C2’	115.9	B	A
l	C5’	115.2	C	
m	C10	103.9	-	D E R
n	C1’’’ (rha)	100.6	G	
o	C1’’ (xyl)	99.5	F	(G) (K)
p	C6	98.7	E	D R
q	C8	93.5	D	E
r	C2’’	77.0	K	
s	C3’’	76.8	N	J
t	C4’’’	71.9	O	Q
u	C2’’’	70.62	I	G
v	C3’’’	70.55	L	G
w	C4’’	69.7	M	(N)
x	C5’’’	68.4	H	G Q
y	C5’’	65.7	J+P	
z	C6’’’	17.4	Q	

While C-H couplings over one bonding are indicated by the HSQC experiment, the HMBC experiment gives couplings over two to four bondings. Thus, most signals could be unambigously assigned. To verify the type of bonding for the anomeric sugar protons as well as the assignment to the C3’’ and C4’’ atom of the xylose moiety, chemical shift rules and several reference studies were consulted (see below).
Furthermore, the assignments of the carbon atoms were in agreement with information from the DEPT spectrum (not presented here).

^{13}C-NMR-Parameters

	δ [ppm] multiplicity J [Hz]		
Assignment	A5 (DMSO-d_6)	Agrawal, 1989; Hamburger *et al.*, 1985 (DMSO-d_6)	Geiger *et al.*, 1983 (DMSO-d_6)
C4	177.2	177.3	177.31
C7	164.2	164.3	164.17
C5	161.2	161.3	161.16
C9	156.2	156.2[a]	156.10[a]
C2	156.1	155.6[a]	156.10[a]
C4’	148.6	148.4	148.48
C3’	145.0	144.9	144.94
C3	132.8	133.2	133.63
C6’	121.5	122.3	122.06
C1’	121.0	121.2	120.81
C2’	115.9	115.6[b]	115.52[b]
C5’	115.2	115.2[b]	115.25[b]
C10	103.9	103.8	103.82
C1’’’ (rha)	100.6		99.94
C1” (xyl)	99.5	99.5	
C6	98.7	98.6	98.65
C8	93.5	93.4	93.42
C2”	77.0	76.6[c]	
C3”	76.8	76.1[c]	
C4’’’	71.9		71.86
C2’’’	70.62		70.55[c]
C3’’’	70.55		70.55[c]
C4”	69.7	69.4	
C5’’’	68.4		68.55
C5”	65.7	65.7	
C6’’’	17.4		17.41

[a – c] Assignments bearing the same superscript may be reversed.

The reference compound given by Hamburger *et al.* (1985) is the quercetin-3-O-(2”-O-ß-D apiofuranosyl-ß-D-xylopyranoside) (= quercetin-3-O-ß-D-apiofuranosyl-(1→2)ß-D-xylopyranoside) with the xylose moiety being directly attached to the quercetin aglycone. Hence, the linkage between the quercetin and the xylose moiety is comparable to the linkage in novel substance A5. It can be stated that the shift values given by Hamburger *et al.* (1985) for the aglycone and the xylose moiety are in agreement with those found for the A5. The assignments of the C3’’ atom and the C4’’ atom are also in agreement with further references on xylose moieties (Bock and Thoegersen, 1982) and their assignments can thus be deduced to the xylose moiety in the novel compound A5.

Geiger *et al.* (1983) described a terminal rhamnose moiety of a disaccharide unit being attached to quercetin: quercetin-3-O-α-L-(2-O-α-L-rhamnopyranosyl-arabinopyranoside).

As the flavonol A5 with its disaccharide has been isolated for the first time, only reference substances could be found with a xylosyl (1→2) rhamnoside moiety, the xylose residue being terminal (Fukai and Nomura, 1988; Mizuno *et al.*, 1988; Slowing *et al.*, 1994).

18 Fraction A1

quercetin glycoside — Proposed formula $C_{33}H_{40}O_{20}$

Proposed structure: quercetin + 1 hexose moiety + 2 deoxyhexose (rhamnose) moieties — MW 756

HPLC

mean retention time	UV-Data λ_{max} / nm			
23.2 min.	255	sh 267	sh 293	354

EI-MS

m/z 302 (54 %) $C_{15}H_{10}O_7$: $[M]^{+\bullet}$, quercetin aglycone
m/z 167 (11 %)
m/z 147 (23 %)
m/z 137 (18 %) $C_7H_5O_3$: $[B_2]^+$
m/z 128 (73 %)
m/z 113 (27 %)
m/z 85 (63 %)
m/z 73 (64 %)
m/z 57 (100 %)
impurity:
m/z 286 (7 %) $C_{15}H_{10}O_6$: $[aglycone]^{+\bullet}$, kaempferol or luteolin
m/z 229 (6 %) $C_{13}H_9O_4$: $[286\text{- CO - CO - H}]^+$

ESI-MS:

positive mode:
m/z 779 (47 %) : $[M + Na]^+$
m/z 499 (100 %) : contamination
m/z 303 (76 %) : $[quercetin + H]^+$
negative mode:
m/z 755 (100 %) : $[M - H]^-$
m/z 301 (31 %) : $[quercetin - H]^-$
impurity:
m/z 739 (27 %) : $[M - H]^-$, assumed as kaempferol or luteolin glycoside

Complementary information of both the positive and negative ESI-mode together with information of the aglycone taken from the EI-mass-spectrum, indicate a molecular weight of 756 Da for a quercetin glycoside. The UV-spectrum corroborates the presence of a quercetin glycoside. The impurity, exhibiting a molecular weight of 740 Da, possesses a luteolin or kempferol moiety as aglycone.

Based on the ^{1}H NMR-spectrum (not depicted here due to poor resolution) there are hints to the occurrence of one hexose and two rhamnose moieties (two doublets at 0.80 and 1.05 ppm with J ca. 6.2 Hz each). Hence, the structure was proposed as indicated above.

19 Fraction A3

Fraction A3 consists primarily of a mixture of three flavonoids. Two compounds were identified as quercetin derivatives, while the third flavonoid could not be further elucidated. The UV-spectra of both quercetin derivatives are very similar and according to mass spectra, the molecular weights differ by just 162 mass units, which corresponds to one hexose moiety. Except for the mass difference, both quercetin compounds equal in their constituents: the same aglycone, one hexose and one deoxyhexose moiety, respectively. The quercetin glycoside exhibiting a molecular weight of 610 Da seems to be the same compound as identified within fraction A2.

quercetin glycoside; A3-1 (M_1)		Formula	$C_{33}H_{40}O_{21}$
Structure	quercetin + 2 hexose moieties + 1 deoxyhexose moiety	MW	772
quercetin glycoside; A3-2 (M_2)		Formula	$C_{27}H_{30}O_{16}$
Structure	quercetin + 1 hexose moiety + 1 deoxyhexose moiety	MW	610

HPLC

mean retention time	UV-Data λ_{max} / nm			
23.4 min.	sh 247	266	sh 293	349
24.0 min. (a quercetin glycoside)	255	sh 267	sh 294	355
24.2 min. (a quercetin glycoside)	255	sh 267	sh 294	354

The quercetin glycoside at 24.0 min. builds the main compound of fraction A3.

EI-MS

The fragmentation pattern of the EI-spectrum of fraction A3 resembles that of fraction A1. Mass m/z 302 with a relative abundance of 47 % can be assigned to quercetin aglycone which is in accordance with expectations from UV-spectra. According to UV-spectra, the main compound of fraction A3 is a quercetin glycoside. As impurity mass m/z 286 with a relative abundance of 20 % might be assigned to a flavone aglycone or kaempferol, which cannot be unambiguously resolved from the corresponding UV-spectrum. Base peak in the EI-spectrum is m/z 57.

ESI-MS

positive mode:

m/z 795 (32 %)	:	$[M_1 + Na]^+$
m/z 633 (100 %)	:	$[M_2 + Na]^+$
MS/MS of mass 795 (M_1)		
m/z 633 (82 %)	:	$[M_1$ - hexose moiety + Na$]^+$
m/z 487 (100 %)	:	$[M_1$ - hexose moiety - deoxyhexose moiety + Na$]^+$
MS/MS of mass 633 (M_2)		
m/z 487 (49 %)	:	$[M_2$ - hexose moiety - deoxyhexose moiety + Na$]^+$
m/z 331 (100 %)	:	$[M_2$ - hexose moiety - quercetin aglycone + Na$]^+$, i.e. m/z 633 minus quercetin moiety (m/z 302).

negative mode:

m/z 771 (54 %)	:	$[M_1 - H]^-$
m/z 609 (100 %)	:	$[M_2$ - H - hexose moiety$]^-$
MS/MS of mass 771 (M_1)		
m/z 609 (100 %)	:	$[M_1$ - H - hexose moiety$]^-$
m/z 463 (6 %)	:	$[M_1$ - H - hexose moiety - deoxyhexose moiety$]^-$
m/z 301 (4 %)	:	$[M_1$ - H - hexose moiety - deoxyhexose moiety - hexose moiety$]^-$, i.e. [quercetin - H$]^-$
MS^3 of mass 609 (M_1)		
m/z 301 (100 %)	:	[quercetin - H$]^-$
MS^3 of mass 463 (M_1)		
m/z 301 (100 %)	:	$[M_1$ - H - 162 - 146 - 162$]^-$, i.e. [quercetin - H$]^-$
MS/MS of mass 609 (M_2)		
m/z 463 (7 %)	:	$[M_2$ - H - hexose moiety – deoxyhexose moiety$]^-$
m/z 301 (100 %)	:	[quercetin - H$]^-$

20 Fraction A6

Fraction A6 contains two substances,
a kaempferol glycoside as the main compound and a quercetin glycoside as a minor component.

kaempferol glycoside; A6-1 (M_1) MW 594

quercetin glycoside; A6-2 (M_2) MW 564

HPLC

mean retention time	UV-Data λ_{max} / nm			
27.3 min. (kaempferol glycoside)	265	sh 293	sh 318 (weak)	348
27.8 min. (quercetin glycoside)	255	sh 267	sh 295	355

The peak height of the kaempferol glycoside is nearly double the height of the subsequent quercetin glycoside within this fraction.

EI-MS

m/z 302 (38 %) $C_{15}H_{10}O_7$:		$[M]^{+\bullet}$, quercetin aglycone
m/z 286 (93 %) $C_{15}H_{10}O_6$:		$[M]^{+\bullet}$, kaempferol aglycone
m/z 267 (48 %)	:	unknown compound, as evidenced from the ion chromatogram
m/z 229 (9 %)		
m/z 180 (8 %)		
m/z 150 (19 %) $C_8H_6O_3$:		$[B_1]^{+\bullet}$, quercetin fragment
m/z 137 (23 %) $C_7H_5O_3$:		$[B_2]^+$, quercetin fragment
m/z 128 (66 %)		
m/z 110 (49 %)		
m/z 85 (63 %)		
m/z 73 (59 %)		
m/z 57 (100 %)		

ESI-MS

positive mode:

m/z 701	(100 %)	:	unknown compound
m/z 617	(13 %)	:	$[M + Na]^+$, sodium adduct of mass 594
m/z 595	(33 %)	:	$[M + H]^+$
m/z 587	(19 %)	:	$[M + Na]^+$, sodium adduct of mass 564
m/z 303	(12 %)	:	$[\text{quercetin} + H]^+$, aglycone
m/z 287	(15 %)	:	$[\text{kaempferol} + H]^+$, aglycone

negative mode:

m/z 593	(33 %)	:	$[M - H]^-$, molecule ion of mass 594
m/z 563	(100 %)	:	$[M - H]^-$, molecule ion of mass 564
m/z 301	(39 %)	:	$[\text{quercetin} - H]^-$, aglycone
m/z 285	(17 %)	:	$[\text{kaempferol} - H]^-$, aglycone

The base peak of the positive ESI-mode is m/z 701, which does not find any counterpart in the mass spectrum of the negative ESI mode. However, there was a range of masses between 10 % and 40 % relative abundance, which can be identified by complementary information. In accordance, one compound can be deciphered with a molecular weight of 594, which is the same as that found in kaempferol-rhamnopyranosyl-galactopyranoside which was contained in fraction A4 (No.9). Despite different retention times, the UV-spectrum of the main compound of fraction A6 resembles that of fraction A4 and both show identical molecular weights. The other component of fraction A6 can be assigned to a molecular weight of 564. In accordance with its UV-spectrum and the occurrence of quercetin as aglycone in the mass spectra it is concluded to be a quercetin glycoside.

21 Fraction A7

Fraction A7 contains several compounds with a kaempferol glycoside as the main compound.

kaempferol glycoside; A7-1 (M_1)		Formula	$C_{27}H_{30}O_{14}$
Structure	kaempferol + 2 deoxyhexose moieties; main compound	MW	578
A7-2 (M_2)		MW	540
quercetin glycoside A7-3 (M_3)		Formula	$C_{22}H_{14}O_{11}$
Structure	quercetin + 1 gallic acid moiety	MW	454

HPLC

mean retention time	UV-Data λ_{max} / nm			
28.8 min. (kaempferol derivative)	265	sh 293	sh 318 (weak)	348

EI-MS

The electron impact mass spectrum yields two aglyca:

m/z 286 (51 %) $C_{15}H_{10}O_6$: $[M]^{+\bullet}$, kaempferol aglycone

impurity:

m/z 302 (8 %) : $[\text{quercetin}]^{+\bullet}$, aglycone

m/z 302 and m/z 286 occurred at the same time in the ion chromatogram.

Base peak in this spectrum is m/z 73.

ESI-MS

positive mode:

m/z 601	(100 %)	:	$[M_1 + Na]^+$
m/z 563	(62 %)	:	$[M_2 + Na]^+$
m/z 455	(40 %)	:	$[M_3 + H]^+$
m/z 169	(37 %)	:	$[146 + 23]^+$, i.e. [deoxyhexose moiety + Na]$^+$
MS/MS of mass 601 (M_1)			
m/z 455 (9 %)		:	$[601 - 146]^+$, i.e. minus deoxyhexose moiety
m/z 315 (100 %)		:	$[601 - 286]^+$, i.e. [M_1 - aglycone moiety + Na]$^+$
MS^3 of mass 315 (M_1)			
m/z 169 (100 %)		:	$[315 - 146]^+$, i.e. minus deoxyhexose moiety; hence [deoxyhexose moiety + Na]$^+$ remains.
MS/MS of mass 563 (M_2)			
m/z 545 (81 %)		:	$[M_2 - H_2O + Na]^+$
m/z 479 (100 %)		:	$[563 - 84]^+$
m/z 394 (48 %)		:	$[563 - 169]^+$, i.e. minus (deoxyhexose moiety + Na^+)
MS/MS of mass 455 (M_3)			
m/z 303 (100 %)		:	$[455 - 152]^+$, i.e. minus gallic acid moiety, resulting in [quercetin aglycone + H]$^+$

negative mode:

m/z 577 (100 %)		:	$[M - H]^-$

Additional MS/MS and MS^3 experiments on the ion m/z 577 did not yield further information and were not documented.

Fraction A7 contains several compounds, all in very low amounts. According to HPLC, a kaempferol glycoside builds the main component. By means of mass spectrometry three molecular weights could be deciphered, 578, 540 and 454 Da, respectively.

From the preceding experiments it can be concluded, that compound M_1 (m/z 578) consists of two deoxyhexose moieties and kaempferol as aglycone. This compound agrees with the UV-spectrum detected during the HPLC run. M_2 (m/z 540) obviously contains a deoxyhexose moiety but cannot be further determined. Compound M_3 (m/z 454) consists of a quercetin aglycone which is attached to a galloyl residue.

22 Fractions A13 and A21

A13

The HPLC chromatogram of fraction A13 shows one main compound and several minor compounds. The UV spectrum as well as the ^{1}H NMR-spectrum indicate that a quercetin glycoside was the main component. By means of mass spectrometry two quercetin glycosides were detected (MW 594 and 448). While M_1 (m/z 594) consists of two deoxyhexose moieties, M_2 (m/z 448) contains one. As the UV-spectrum can be assigned to M_1, this must be the main compound. A third substance shows a molecular weight of 418 Da, which belongs to a kaempferol aglycone, which in turn is linked to a pentose moiety.

quercetin glycoside; A13-1 (M_1)		Formula	$C_{27}H_{30}O_{15}$
Structure	quercetin aglycone + 2 deoxyhexose moieties; main compound	MW	594

quercetin glycoside; A13-2 (M_2)		Formula	$C_{21}H_{20}O_{11}$
Structure	quercetin + 1 deoxyhexose moiety	MW	448
kaempferol glycoside; A13-3 (M_3)		Formula	$C_{20}H_{18}O_{10}$
Structure	kaempferol + 1 pentose moiety	MW	418

HPLC

mean retention time	UV-Data λ_{max} / nm			
28.8 min. (quercetin glycoside, main compound)	255	sh 267	sh 294	356

A21

Fraction A21 contains two components: a quercetin glycoside as major compound and a kaempferol glycoside as minor compound.

quercetin glycoside; A21-1 (M_2)		Formula	$C_{21}H_{20}O_{11}$
Structure	quercetin + 1 deoxyhexose moiety	MW	448
kaempferol glycoside; A21-2 (M_1)		Formula	$C_{20}H_{18}O_{10}$
Structure	kaempferol + 1 pentose moiety	MW	418

HPLC

mean retention time	UV-Data λ_{max} / nm			
28.6 min. (quercetin glycoside)	255	sh 267	sh 293	355
28.7 min. (kaempferol glycoside)	264	sh 293	sh 318 (weak)	348

Both fractions, A13 and A21, contain a quercetin glycoside (MW 448) and a kaempferol glycoside (MW 418), respectively. As both glycosides occur only in trace amounts in fraction A13 (for which retention times have therefore not been recorded), HPLC data on these compounds are only available for fraction A21. However, due to their close sequence in both fractions it is reasonable to assume that both fractions contain identical compounds. As fragmentation patterns in the EI mode as well as data obtained from ESI experiments (including MS/MS and MS^3 experiments) are largely the same, only data from fraction A13 are presented here.

Regarding the glycoside identified with a molecular weight of 448, it is necessary to point out that neither its UV spectrum, nor its retention time agree with data obtained for quercitrin (A12), although the corresponding molecular weights are identical. Therefore, it can be concluded that the quercitrin and the quercetin glycoside of fractions A13 / A21 are constitutional isomers.

EI-MS (A13)

quercetin

m/z 302	(63 %)	$C_{15}H_{10}O_7$:	$[M]^{+\bullet}$, quercetin aglycone]
m/z 273	(5 %)	$C_{14}H_9O_6$:	$[M - CO - H]^+$
m/z 258	(11 %)		
m/z 213	(6 %)		
m/z 153	(11 %)	$C_7H_5O_4$:	$[A_1 + H]^+$, occurring for both aglyca
m/z 137	(14 %)	$C_7H_5O_3$:	$[B_2]^+$, quercetin fragment
m/z 128	(17 %)		
m/z 73	(23 %)		
m/z 69	(26 %)		

m/z 57 (30 %)

kaempferol

m/z 286 (100 %) $C_{15}H_{10}O_6$: [kaempferol]$^{+\bullet}$, aglycone

m/z 229 (14 %) $C_{13}H_9O_4$: [286 - CO - CO - H]$^+$

m/z 121 (19 %) $C_7H_5O_2$: [B_2]$^+$, kaempferol fragment

ESI-MS (A13)

positive mode

m/z 617 (10 %) : [M_1 + Na]$^+$

m/z 487 (27 %) : [M_2 + K]$^+$

m/z 471 (100 %) : [M_2 + Na]$^+$

m/z 441 (36 %) : [M_3 + Na]$^+$

m/z 169 (2 %) : [deoxyhexose moiety + Na]$^+$

MS/MS of mass 617 (M_1)

m/z 533 (100 %) : [617 - 84]$^+$

m/z 471 (14 %) : [M_1 - deoxyhexose moiety + Na]$^+$

m/z 325 (3 %) : [quercetin aglycone + Na]$^+$

m/z 315 (54 %)

MS^3 of mass 533 (M_1)

m/z 487 (100 %) : [M_1 - deoxyhexose moiety + K]$^+$

m/z 415 (43 %)

m/z 341 (6 %) : [quercetin aglycone + K]$^+$

MS/MS of mass 471 (M_2)

m/z 325 (100 %) : [M_2 - deoxyhexose moiety + Na]$^+$, i.e. [quercetin aglycone + Na]$^+$

m/z 303 (5 %) : [quercetin aglycone + H]$^+$

m/z 169 (14 %) : [deoxyhexose moiety + Na]$^+$

MS/MS of mass 441 (M_3)

m/z 309 (100 %) : [M_3 - pentose moiety + Na]$^+$, i.e. [kaempferol aglycone + Na]$^+$

m/z 155 (6 %) : [pentose moiety + Na]$^+$, i.e. [132 + 23]$^+$

negative mode:

m/z 895 (21 %) : [2 x 448 - H]$^-$

m/z 593 (22 %) : [M_1 - H]$^-$

m/z 447 (100 %) : [M_2 - H]$^-$

m/z 417 (15 %) : [M_3 - H]$^-$

MS/MS of mass 593 (M_1)

m/z 447 (19 %) : [M_1 - deoxyhexose moiety - H]$^-$

m/z 301 (100 %) : [M_1 - 2 deoxyhexose moieties - H]$^-$, i.e. [quercetin aglycone - H]$^-$

MS^3 of mass 447 (M_1)

m/z 301 (100 %) : [quercetin aglycone - H]$^-$

MS/MS of mass 447 (M_2)

m/z 301 (100 %) : [M_2 - deoxyhexose moiety - H]$^-$, i.e. [quercetin aglycone - H]$^-$

MS/MS of mass 417 (M_3)

m/z 285 (100 %) : [M_3 - pentose moiety - H]$^-$, i.e. [kaempferol aglycone - H]$^-$

23 Fraction A20

quercetin glycoside Formula $C_{20}H_{18}O_{11}$

Structure quercetin + 1 pentose moiety MW 434

HPLC

mean retention time	UV-Data λ max / nm
26.3 min.	255 sh 266 sh 293 355

EI-MS

m/z 302 (100 %) $C_{15}H_{10}O_7$: $[M]^{+\bullet}$, quercetin
m/z 285 (1 %) $C_{15}H_9O_6$: $[M - OH]^+$
m/z 273 (4 %) $C_{14}H_9O_6$: $[M - CO - H]^+$
m/z 257 (2 %)
m/z 245 (4 %) $C_{13}H_9O_5$: $[M - CO - CO - H]^+$
m/z 228 (3 %)
m/z 153 (5 %) $C_7H_5O_4$: $[A_1 + H]^+$
m/z 137 (7 %) $C_7H_5O_3$: $[B_2]^+$

ESI-MS:

positive mode:
m/z 457 (56 %) : $[M + Na]^+$
m/z 303 (100 %) : $[\text{quercetin} + H]^+$

negative mode:
m/z 433 (100 %) : $[M - H]^-$
m/z 301 (43 %) : $[\text{quercetin} - H]^-$

The molecular weight can be determined as 434 Da by complementary information of the ESI spectra. The aglycone is corroborated by the EI mass spectrum and expected from the UV spectra. Hence, fraction A20 contains a quercetin glycoside with a molecular weight of 434. The mass difference of m/z 132 between the molecular weight and the aglycone pleads for a pentose moiety (presumably xylose) being attached to the quercetin aglycone.

24 Fraction R7

acylated auronol glycoside; R7-1 (M_1) Formula $C_{28}H_{26}O_{15}$

Structure aglycone (auronol) + 1 gallic acid moiety + 1 hexose moiety MW 602

gallic acid derivative; R7-2 (M_2) Formula $C_{20}H_{20}O_{14}$

Structure 2 gallic acid moieties + 1 hexose moiety, presumably digalloylglucose MW 484

HPLC

mean retention time	UV-Data λ max / nm
19.1 min.	216 276

The UV spectrum pleads for a gallic acid derivative, which is assigned to M_2, the digalloylhexose.

EI-MS

m/z 184	(43 %)	$C_8H_8O_5$:	$[M]^{+\bullet}$, gallic acid methyl ester
m/z 170	(39 %)	$C_7H_6O_5$:	$[M]^{+\bullet}$, gallic acid
m/z 153	(61 %)	$C_7H_5O_4$:	$[M - OH]^+$, according to ion traces originating from gallic acid
m/z 125	(30 %)	$C_6H_5O_3$:	pyrogallol cation
m/z 110	(21 %)		
m/z 97	(37 %)		
m/z 85	(31 %)		
m/z 69	(60 %)		
m/z 55	(100 %)		

The EI mass spectrum identified masses of gallic acid (m/z 170) and gallic acid methyl ester (m/z 184) and the corresponding typical fragmentation scheme. Their common fragment is pyrogallol. Base peak is m/z 55.

ESI-MS

positive mode:

m/z 625	(19 %)	:	$[M_1 + Na]^+$
m/z 523	(28 %)	:	$[M_2 + K]^+$
m/z 507	(100 %)	:	$[M_2 + Na]^+$
m/z 485	(6 %)	:	$[M_2 + H]^+$
MS/MS of mass 625 (M_1)			
m/z 549	(28 %)		
m/z 473	(100 %)	:	$[M_1$ - gallic acid moiety + Na$]^+$
m/z 337	(62 %)	:	$[M_1$ - aglycone (288) + Na$]^+$, hence $[314 + Na]^+$ remains
m/z 319	(13 %)	:	$[314 + Na - H_2O]^+$
MS^3 of mass 337 (M_1)			
m/z 319	(100 %)	:	$[314 + Na - H_2O]^+$
m/z 259	(21 %)	:	$[314 + Na - 18 - 60]^+$
m/z 185	(2 %)	:	$[314 + Na$ - gallic acid moiety$]^+$
m/z 175	(12 %)	:	$[314 + Na$ - hexose moiety$]^+$
MS/MS of mass 507 (M_2)			
m/z 337	(100 %)	:	$[M_2 + Na$ - gallic acid$]^+$, $[484 + 23 - 170]^+$
MS^3 of mass 337 (M_2)			
m/z 175	(100 %)	:	$[314 + Na$ - hexose moiety$]^+$

negative mode:

m/z 601	(37 %)	:	$[M_1 - H]^-$
m/z 483	(100 %)	:	$[M_2 - H]^-$
MS/MS of mass 601 (M_1)			
m/z 449	(100 %)	:	$[M_1$ - gallic acid moiety - H$]^-$
m/z 287	(27 %)	:	$[M_1$ - gallic acid moiety - hexose moiety - H$]^-$, i.e. [aglycone (288) - H$]^-$
m/z 259	(11 %)	:	$[M_1$ - gallic acid moiety - hexose moiety - CO - H$]^-$
MS^3 of mass 449 (M_1)			
m/z 287	(7 %)	:	[aglycone - H$]^-$
m/z 271	(100 %)		
m/z 211	(32 %)		
m/z 169	(74 %)	:	[gallic acid - H$]^-$

MS^3 of mass 287 (M_1)			
m/z 259	(100 %)	:	[aglycone - CO - H]$^-$
MS/MS of mass 483 (M_2)			
m/z 423	(12 %)	:	[483 - 60]$^-$, i.e. [M_2 - H - α-hydroxy-acetaldehyde]$^-$
m/z 331	(27 %)	:	[483 - gallic acid moiety (152)]$^-$
m/z 313	(28 %)	:	[483 - gallic acid (170)]$^-$
m/z 271	(100 %)	:	[483 - 60 - 152]$^-$
m/z 211	(17 %)	:	[271 – 60]$^-$, i.e. [271 - 1,2-dihydroxy-ethene]$^-$
m/z 169	(12 %)	:	[gallic acid - H]$^-$
MS^3 of mass 313 (M_2)			
m/z 169	(100 %)	:	[gallic acid - H]$^-$
m/z 125	(18 %)	:	[pyrogallol - H]$^-$

From the ESI experiments it is concluded that in fraction R7, which contains several compounds, two substances can be assigned to a molecular weight of 602 (M_1) and 484 (M_2), respectively. The ESI spectra of both compounds contain a fragment of m/z 337, consisting of a gallic acid moiety and a hexose moiety. In the case of M_1 it can be stated that the sugar moiety was esterified by gallic acid and this unit is attached to the aglycone of mass 288, presumably an auronol (compare especially fragment m/z 337 and its further fragmentation in the positive mode). M_2 is concluded to be a digalloylhexose, presumably digalloylglucose. Both, the positive and negative mode indicate a hexose (presumably glucose) which is esterified by two galloyl residues at two different positions except for the hydroxyl function at C6 of the sugar moiety, which in turn is evidenced by neutral loss of 60 Dalton (α-hydroxy-acetaldehyde) in the negative mode.

25 Fraction R9

acylated auronol glycoside; R9-1 (M_1)		Formula	$C_{28}H_{26}O_{15}$
Structure	auronol (e.g. maesopsin) + 1 gallic acid moiety + 1 hexose moiety	MW	602
R9-2 (M_2)		MW	630

HPLC

mean retention time	UV-Data λ $_{max}$ / nm		
26.1 min.	211	290	sh 338

EI-MS

m/z 184	(83 %)	$C_8H_8O_5$:	$[M]^{+\bullet}$, gallic acid methyl ester
m/z 153	(100 %)	$C_7H_5O_4$: - $OCH_3^\bullet$ →	$[M - OCH_3]^+$
m/z 120			
m/z 107	(76 %)	$C_6H_3O_2$:	$[M - OCH_3 - CO - H_2O]^+$
m/z 91	(49 %)	C_7H_7:	benzyl cation ↔ tropylium ion
m/z 73	(46 %)		
m/z 60	(73 %)		

The EI spectrum shows the typical fragmentation pattern of gallic acid methyl ester.

ESI-MS

positive mode:

m/z 625	(100 %)	:	$[M_1 + Na]^+$

impurity:

m/z 669 (6 %)	:	$[M_2 + K]^+$
m/z 653 (14 %)	:	$[M_2 + Na]^+$

MS/MS of mass 625 (M_1)

m/z 607 (7 %)	:	$[M_1 - H_2O + Na]^+$
m/z 463 (100 %)	:	$[M_1$ - hexose moiety $+ Na]^+$

MS^3 of mass 463 (M_1)

m/z 445 (100 %)	:	$[463 - H_2O]^+$
m/z 435 (72 %)	:	$[463 - CO]^+$

negative mode:

m/z 601 (100 %)	:	$[M_1 - H]^-$

impurity:

m/z 629 (15 %)	:	$[M_2 - H]^-$

MS/MS of mass 601 (M_1)

m/z 601 (7 %)	:	$[M_1 - H]^-$
m/z 583 (13 %)	:	$[M_1 - H_2O - H]^-$
m/z 439 (100 %)	:	$[M_1$ - hexose moiety $- H]^-$
m/z 421 (19 %)	:	$[439 - H_2O]^-$
m/z 287 (6 %)	:	$[439$ - gallic acid moiety$]^-$, i.e. $[$aglycone $- H]^-$
m/z 269 (39 %)	:	$[287 - H_2O]^-$

MS^3 of mass 439 (M_1)

m/z 421 (21 %)	:	$[439 - H_2O]^-$
m/z 287 (18 %)	:	$[439$ - gallic acid moiety$]^-$
m/z 269 (100 %)	:	$[287 - H_2O]^-$
m/z 259 (6 %)	:	$[287 - CO]^-$
m/z 243 (4 %)	:	$[287 - CO_2]^-$

Fraction R9 is shown to contain a compound with a molecular weight of 602, which consists of a hexose moiety, a gallic acid moiety and an aglycone of 288 Da, based on UV spectrum an auronol, presumably maesopsin.
As fragments observed under MS/MS conditions differ from those of fraction R7, it is concluded that both compounds are positional isomers. In compound R9 both the galloyl and the hexose residue are attached to different positions of the aglycone.
The impurity in this fraction is a compound with a molecular weight of 630.

26 Fraction R13, R14

According to UV-spectra and data obtained by mass spectrometry, both fractions contain gallic acid derivatives. Based on NMR experiments, these compounds are isomers.

gallic acid derivatives

proposed structure	gallic acid (MW 170) + mass difference of 156 u	MW	326

HPLC

mean retention time	UV-Data λ_{max} / nm	
16.3 min. (R13)	215	274
16.6 min. (R14)	216	275

According to UV-spectra gallic acid derivatives are to be expected.

EI-MS (R13 and R14)

m/z 170 (97% and 54 %) : $[M]^{+\bullet}$, gallic acid
m/z 153 (63 % and 25 %):- $OH^{\bullet} \rightarrow [M - OH]^{+}$
m/z 138 (100 %) : $[156 - H_2O]^{+\bullet}$
m/z 126 (69 % and 90 %): $[\text{pyrogallol}]^{+\bullet}$
m/z 121 (83 % and 81 %): $[138 - OH]^{+}$
m/z 108 (20 % and 22 %)
m/z 94 (44 % and 46 %)
m/z 80 (20 and 25 %)

The EI-MS spectra of both fractions show identical fragmentation patterns with gallic acid (m/z 170) as molecule radical cation, respectively.

ESI-MS

Fraction R14 was used to document the positive mode representatively:
positive mode:

m/z 675 (6 %) : $[2 M + Na]^{+}$
m/z 365 (44 %) : $[M + K]^{+}$
m/z 349 (95 %) : $[M + Na]^{+}$
m/z 107 (100 %)
MS/MS of mass 349
m/z 179 (100 %) : $[156 + Na]^{+}$

Most valuable information could be gained by ESI-experiments in the negative mode. Experiments on fraction R13 are documented representatively:
negative mode:

m/z 651 (83 %) : $[2 M - H]^{-}$
m/z 353 (9 %)
m/z 325 (100 %) : $[M - H]^{-}$
MS/MS of mass 325
m/z 169 (100 %) : $[\text{gallic acid - H}]^{-}$
m/z 125 (9 %) : $[169 - CO_2]^{-}$, i.e. $[\text{pyrogallol - H}]^{-}$
MS^3 of mass 169 corroborates that fragment m/z 125 originates from m/z 169.
MS/MS of mass 353
m/z 307 (98 %) : $[353 - 46]^{-}$
m/z 289 (45 %) : $[353 - 46 - 18]^{-}$
m/z 245 (49 %) : $[353 - 46 – 18 - 44]^{-}$
m/z 201 (48 %) : $[353 \text{ - gallic acid moiety}]^{-}$
m/z 169 (100 %) : $[\text{gallic acid - H}]^{-}$
m/z 125 (22 %) : $[\text{pyrogallol - H}]^{-}$, pyrogallol anion

Both fractions R13 and R14 contain a compound with a molecular weight of 326, which consists of gallic acid (170 Da) as integral constituent and a mass difference of 156 units. The UV-spectra are nearly identical and also indicate for gallic acid derivatives. NMR experiments (not depicted here) indicate two isomeric compounds.

27 Fraction R17

acylated quercetin glycoside Formula $C_{28}H_{24}O_{16}$

Structure quercetin + 1 gallic acid moiety MW 616
+ 1 hexose moiety

HPLC

mean retention time	UV-Data λ_{max} / nm
25.1 min.	262 sh 293 354

Mass spectrometry

In the EI-MS spectrum a quercetin aglycone can be observed as well as masses that are assumed to originate from gallic acid. In case of gallic acid, this is explicitly noted. However, other masses are identified as fragments of quercetin.

EI-MS

m/z 302 (100 %) $C_{15}H_{10}O_7$: $[M]^{+\bullet}$, quercetin (aglycone)
m/z 274 (6 %) $C_{14}H_{10}O_6$: $[M - CO]^{+\bullet}$
m/z 170 (7 %) $C_7H_6O_5$: $[\text{gallic acid}]^{+\bullet}$
m/z 153 (37 %) $C_7H_5O_4$: $[A_1 + H]^+$
m/z 137 (9 %) $C_7H_5O_3$: $[B_2]^+$
m/z 107 (11 %) $C_6H_3O_2$: $[\text{gallic acid - OH- CO - } H_2O]^+$
m/z 69 (6 %)

ESI-MS

positive mode:
m/z 655 (19 %) : $[M + K]^+$, quercetin glycoside
m/z 639 (100 %) : $[M + Na]^+$, quercetin glycoside
m/z 487 (4 %) : $[639 \text{ - gallic acid moiety}]^+$
MS/MS of mass 639:
m/z 485 (10 %)
m/z 337 (100 %) : $[M \text{ - quercetin} + Na]^+$
m/z 325 (10 %) : $[\text{aglycone} + Na]^+$, quercetin
m/z 303 (2 %) : $[\text{aglycone} + H]^+$, quercetin
negative mode:
m/z 615 (100 %) : $[M - H]^-$, quercetin glycoside
MS/MS of mass 615
m/z 463 (100 %) : $[615 \text{ - galloyl moiety}]^-$
m/z 301 (20 %) : $[615 \text{ - galloyl moiety - hexose moiety}]^-$, i.e. $[\text{quercetin aglycone - H}]^-$

From mass spectrometry it is reasonable to assume a quercetin glycoside with a hexose moiety as a sugar component, and a gallic acid moiety.

28 Fraction R18

Fraction R18 obviously contains one major compound, which was assigned to a kaempferol glycoside, and three minor glycosides (for quercetin contained see A15, A16).

acylated dihydrokaempferol glycoside; R18-1 (M_1) Formula $C_{29}H_{28}O_{15}$

Structure dihydrokaempferol + 1 hexose moiety + 1 methoxy gallic acid moiety MW 616

kaempferol glycoside; R18-2 (M_2) Formula $C_{28}H_{24}O_{15}$

Structure kaempferol + 1 hexose moiety + 1 gallic acid moiety MW 600

quercetin glycoside; R18-3 (M_3) Formula $C_{21}H_{20}O_{12}$

Structure quercetin + 1 hexose moiety MW 464

kaempferol glycoside; R18-4 (M_4) Formula $C_{21}H_{20}O_{11}$

Structure kaempferol + 1 hexose moiety MW 448

HPLC

mean retention time	UV-Data λ_{max} / nm
26.2 min.	265 sh 293 346

The UV-spectrum is assigned to a kaempferol glycoside as major component.

EI-MS

In the following EI-MS spectrum three flavonoid aglyca are apparent, which are assigned to quercetin (m/z 302) kaempferol (m/z 286) and apigenin (m/z 270) as aglyca. As evidenced by ESI experiments, the apigenin aglycone originates from dihydrokaempferol, which rearranges to apigenin under experimental conditions (see Fig. II.7).

The identification of kaempferol is based on the ion chromatogram and the fragmentation pattern of kaempferol in the EI spectrum. In the ion chromatogram, the occurrence of fragment m/z 121 ($[B_2]^+$ ion) is connected to the occurrence of ion m/z 286, which indicates kaempferol as aglycone. According to fragmentation scheme (Fig. C.7, p. 102), the isomeric compound luteolin (m/z 286) shows a different fragmentation pattern with m/z 137 as $[B_2]^+$ ion.

m/z 302 (11 %) $C_{15}H_{10}O_7$: $[M]^{+\bullet}$, quercetin (aglycone)
m/z 286 (100 %) $C_{15}H_{10}O_6$: $[M]^{+\bullet}$, kaempferol (aglycone)
m/z 270 (8 %) $C_{15}H_{10}O_5$: $[M]^{+\bullet}$, apigenin (aglycone)
m/z 229 (5 %) $C_{13}H_9O_4$: [kaempferol- CO - CO - H]$^+$
m/z 184 (5 %) $C_8H_8O_5$: [methoxy gallic acid]$^{+\bullet}$
m/z 170 (5 %) $C_7H_6O_5$: $[M]^{+\bullet}$, gallic acid
m/z 153 (26 %) $C_7H_5O_4$: $[A_1+ H]^+$
m/z 121 (11 %) $C_7H_5O_2$: $[B_2]^+$ originating from kaempferol and / or apigenin
m/z 69 (6 %)
m/z 65 (6 %)
m/z 39 (6 %)

According to ion traces, the fragment m/z 153 originated from the flavonoid aglycon m/z 286 by RDA reaction and is not a fragment of gallic acid. The assignment of m/z 184 to methoxy gallic acid is based on results obtained from ESI experiments.

ESI-MS

positive mode:

m/z 639 (38 %) : $[M_1 + Na]^+$
m/z 623 (100 %) : $[M_2 + Na]^+$
m/z 487 (20 %) : $[M_3 + Na]^+$
m/z 471 (28 %) : $[M_4 + Na]^+$

MS/MS of mass 639 (M_1)

m/z 477 (2 %) : [M_1 - hexose moiety + Na]$^+$
m/z 456 (100 %) : [M_1 - methoxy gallic acid radical + Na]$^{+\bullet}$
m/z 294 (1 %) : [456 - hexose moiety]$^{+\bullet}$

MS/MS of mass 623 (M_2)

m/z 595 (9 %) : $[M_2 - CO + Na]^+$
m/z 477 (5 %)
m/z 471 (2 %) : [M_2 - gallic acid moiety + Na]$^+$
m/z 337 (100 %) : [M_2 - kaempferol aglycone + Na]$^+$, hence [gallic acid moiety + hexose moiety + Na]$^+$ remains.

m/z 309 (8 %) : [M_2 - gallic acid moiety - hexose moiety + Na]$^+$, hence [kaempferol + Na]$^+$ remains.

MS/MS of mass 487 (M_3)

m/z 325 (100 %) : [M_3 - hexose moiety + Na]$^+$, i.e. [quercetin aglycone + Na]$^+$

m/z 185 (57 %) : [hexose moiety + Na]$^+$

MS/MS of mass 471 (M_4)

m/z 309 (76 %) : [M_4 - hexose moiety + Na]$^+$, i.e. [kaempferol aglycone + Na]$^+$

m/z 185 (100 %) : [hexose moiety + Na]$^+$

negative mode:

m/z 615 (47 %) : [M_1 - H]$^-$

m/z 599 (100 %) : [M_2 - H]$^-$

m/z 463 (10 %) : [M_3 - H]$^-$

m/z 447 (6 %) : [M_4 - H]$^-$

MS/MS of mass 615 (M_1)

m/z 463 (7 %) : [M_1 - RDA fragment of dihydrokaempferol - H]$^-$

m/z 453 (20 %) : [M_1 - hexose moiety - H]$^-$, i.e. [615 - 162]$^-$

m/z 431 (100 %) : [M_1 - methoxy gallic acid - H]$^-$, i.e. [615 - 184]$^-$

m/z 269 (3 %) : [apigenin - H]$^-$

MS^3 of mass 453 (M_1)

m/z 269 (100 %) : [M_1 - hexose moiety - methoxy gallic acid - H]$^-$, i.e. [apigenin - H]$^-$, [453 - 184]$^-$

m/z 183 (9 %) : [methoxy gallic acid - H]$^-$, i.e. [453 - 270]$^-$

MS^3 of mass 431 (M_1)

m/z 269 (100 %) : [M_1 - methoxy gallic acid - hexose moiety - H]$^-$, i.e. [apigenin - H]$^-$

MS/MS of mass 599 (M_2)

m/z 447 (100 %) : [M_2 - gallic acid moiety - H]$^-$

m/z 313 (56 %) : [M_2 - kaempferol - H]$^-$

m/z 285 (8 %) : [kaempferol - H]$^-$, which corresponds to [M_2 - gallic acid moiety - hexose moiety - H]$^-$

MS^3 of mass 447 (M_2)

m/z 285 (100 %) : [M_2 - gallic acid moiety - hexose moiety - H]$^-$, i.e. [kaempferol - H]$^-$

MS^3 of mass 313 (M_2)

m/z 169 (100 %) : [gallic acid - H]$^-$

m/z 125 (17 %) : [pyrogallol - H]$^-$

MS/MS of mass 463 (M_3)

m/z 301 (100 %) : [M_3 - hexose moiety - H]$^-$, i.e. [quercetin - H]$^-$

m/z 271 (4 %)

m/z 179 (4 %) : [hexose - H]$^-$

m/z 151 (2 %) : [gallic acid moiety - H]$^-$

MS^3 of mass 301 (M_3)

m/z 183 (100 %)

m/z 151 (69 %) : [gallic acid moiety - H]$^-$

MS/MS of mass 447 (M_4)

m/z 285 (100 %) : [M_4 - hexose moiety - H]$^-$, i.e. [kaempferol or luteolin- H]$^-$

M_1 (MW 616) is concluded to consist of dihydrokaempferol as aglycone, which is esterified at position 3 by a methoxy gallic acid moiety (which is depicted as 4-methoxy gallic acid residue in Fig. II.7), and to which a hexose residue is attached at 4'-position. Despite ESI conditions, the dihydrokaempferol is supposed to decompose yielding apigenin as aglycone (see Fig. II.7). Both, the methoxy gallic acid and the hexose residue may initially leave the molecule ion and then yield to m/z 431 and m/z 453, respectively, as evidenced by MS/MS and MS^3 experiments (ESI conditions, negative mode). Furthermore, the RDA reaction occurs resulting in m/z 463 and a neutral loss of mass 152, $(M_1\text{-H-152})^-$, which corroborates that the aglycone positions 5 and 7 are unsubstituted. Moreover, it is notable to mention that in spite of ESI conditions (positive mode) a methoxy gallic acid radical is produced resulting in a radical cation (m/z 456).

It should also be mentioned that although the preceding fraction R17 contains a compound of the same molecular weight like M_1 of R18, both compounds are completely different.

M_2 (MW 600) consists of kaempferol as aglycone that is linked to a hexose moiety and a gallic acid moiety.

M_3 (MW 464) consists of quercetin as aglycone that is linked to a hexose moiety, while

M_4 (MW 448) contains a kaempferol as aglycone linked to a hexose moiety.

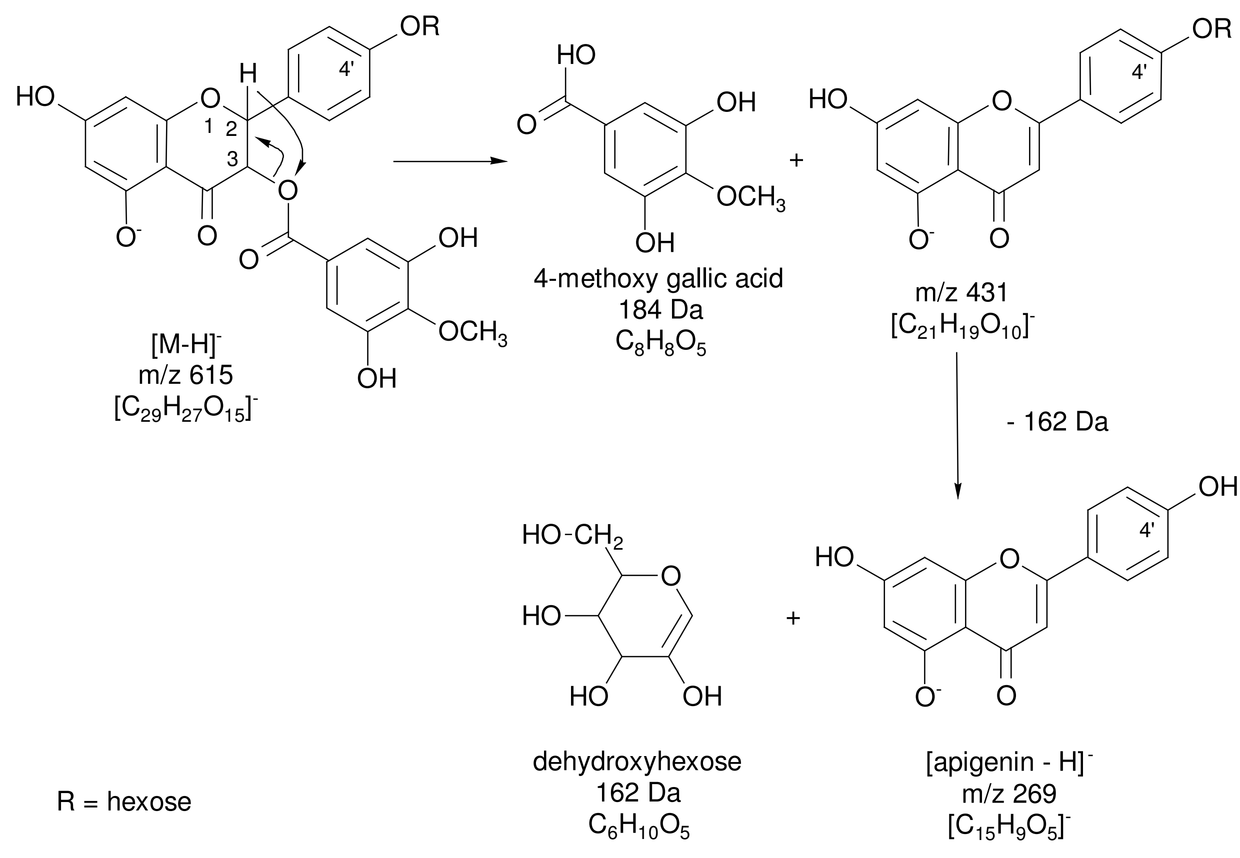


Fig. II.7: Proposed rearrangement and fragmentation of dihydrokaempferol-3-(methoxy gallic acid)-4'-hexose to apigenin (aglycone), methoxy gallic acid and dehydroxyhexose under ESI conditions. (The methoxy gallic acid residue is depicted as 4-methoxy gallic acid.)

29 Fraction R19

kaempferol glycoside; R19-1 (M_1)		Formula	$C_{20}H_{18}O_{10}$
Structure	kaempferol + 1 pentose moiety	MW	418
R19-2 (M_2)		MW	330

HPLC

mean retention time	UV-Data λ_{max} / nm			
29.0 min. (kaempferol glycoside)	253	264	sh 294	347

EI-MS

m/z 286 (100 %) $C_{15}H_{10}O_6$: $[M]^{+\bullet}$, kaempferol (aglycone)
m/z 258 (8 %) $C_{14}H_{10}O_5$: $[M - CO]^{+\bullet}$
m/z 229 (7 %) $C_{13}H_9O_4$: $[M - CO - CO - H]^+$
m/z 153 (25 %) $C_7H_5O_4$: $[A_1 + H]^+$
m/z 121 (20 %) $C_7H_5O_2$: $[B_2]^+$
impurity:
m/z 184 (12 %) : $[M]^{+\bullet}$, presumably gallic acid methyl ester

The molecule radical cation m/z 286 combined with fragment m/z 121 indicate kaempferol as aglycone of the flavonoid compound.

ESI-MS

positive mode:
m/z 441 (65 %) : $[M_1 + Na]^+$
m/z 353 (100 %) : $[M_2 + Na]^+$
MS/MS of mass 441 (M_1)
m/z 309 (100 %) : $[M_1$ - pentose moiety + Na$]^+$
m/z 155 (9 %) : $[M_1$ - kaempferol aglycone + Na$]^+$, i.e. [pentose moiety + Na$]^+$
MS/MS of mass 353 (M_2)
m/z 338 (100 %) : $[M_2 - 15 + Na]^+$
negative mode:
m/z 417 (100 %) : $[M_1 - H]^-$
m/z 329 (50 %) : $[M_2 - H]^-$
MS/MS of mass 417 (M_1)
m/z 285 (40 %) : $[M_1$ - pentose moiety - H$]^-$, i.e. [aglycone - H$]^-$
m/z 284 (100 %)

From complementary information of the positive and negative ESI-spectra, it can be inferred that two compounds occurred in fraction R19. One compound has a mass of 418 Da and consists of a kaempferol aglycone and a pentose moiety. The other substance has a molecular weight of 330 Da and is no flavonoid.

Based on differing UV spectra (with special respect to band II), the kaempferol glycosides of fractions A13/A21 and R19 are concluded to be positional isomers.

30 AP23/24

presumably a gallic acid derivative

HPLC

mean retention time	UV-Data λ_{max} / nm
24.9 min.	277

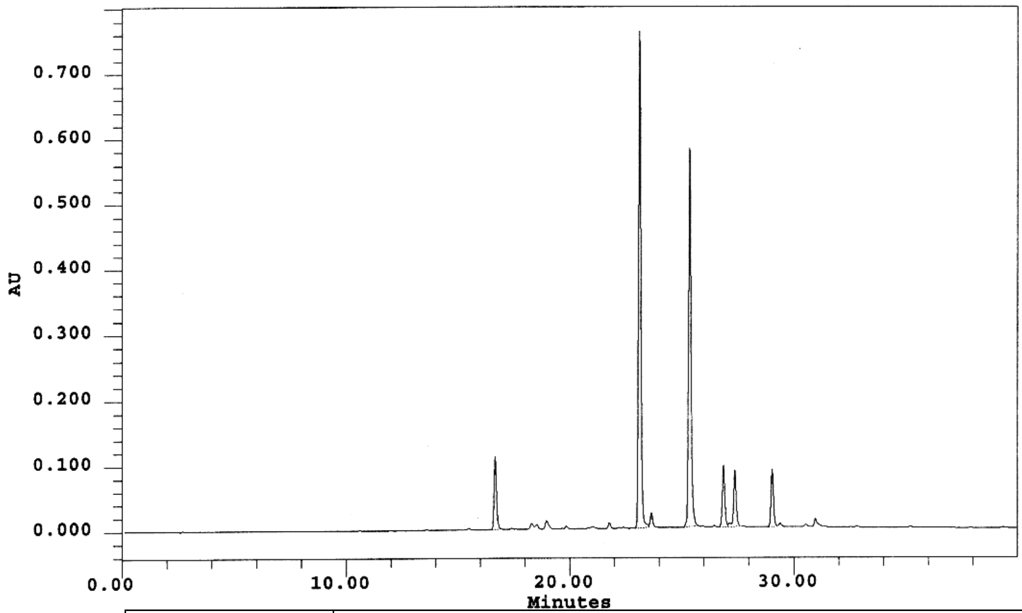
31 AP25

HPLC

mean retention time	UV-Data λ_{max} / nm				
20.0 min.	217	sh 234	sh 243	sh 297	326
22.3 min.	227			sh 297	311
22.7 min.					306
23.7 min.	227			sh 297	312
23.9 min.	sh 226			295	
25.0 min.	sh 223			sh 298	312
48.5 min.	224		274		

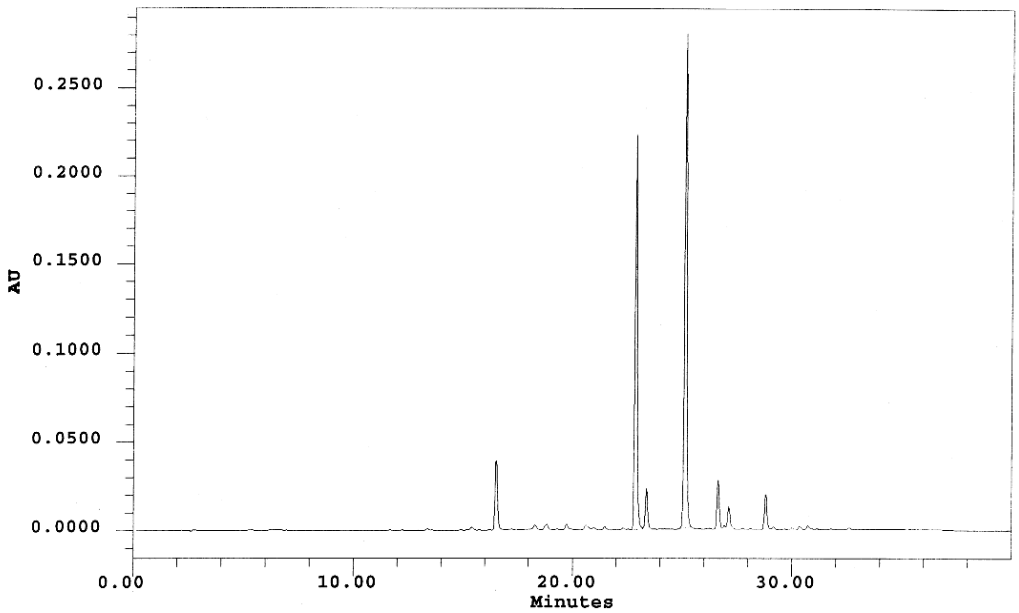
II.4 Review on HPLC chromatograms

In Fig. II.8 to Fig. II.15 HPLC chromatograms are shown of *Cercis canadensis*, *Robinia pseudoacacia*, *Acer rubrum* and *Liquidambar styraciflua* leaves (unhydrolysed leaf extracts) which take into account the seasonal differences of May and October leaves. Only chromatograms showing each species' major group of secondary compounds are depicted here. The wavelength chosen for detection by UV light was dependent upon the major group of characteristic secondary compounds (tannins at 270 nm, flavonoids at 350 nm).



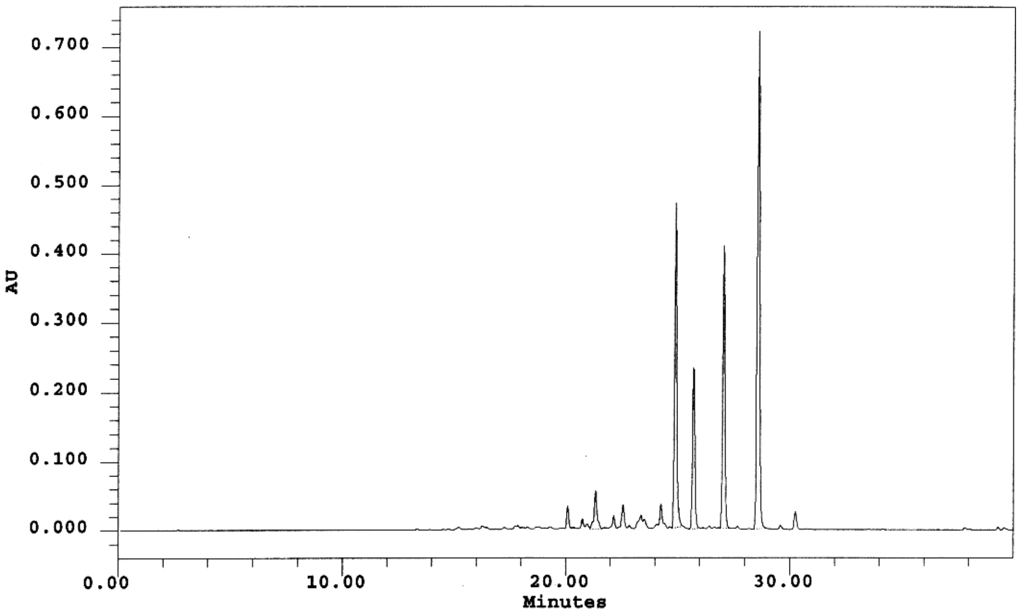
ret. time (min)	name
16.4	dihydroxyflavone (Mabry *et al.*, 1970)
22.9	flavonol
25.1	quercitrin
26.6	flavonoid
27.2	afzelin
28.8	flavonoid

Fig. II.8: *Cercis canadensis*: May leaves (detection at 350 nm).



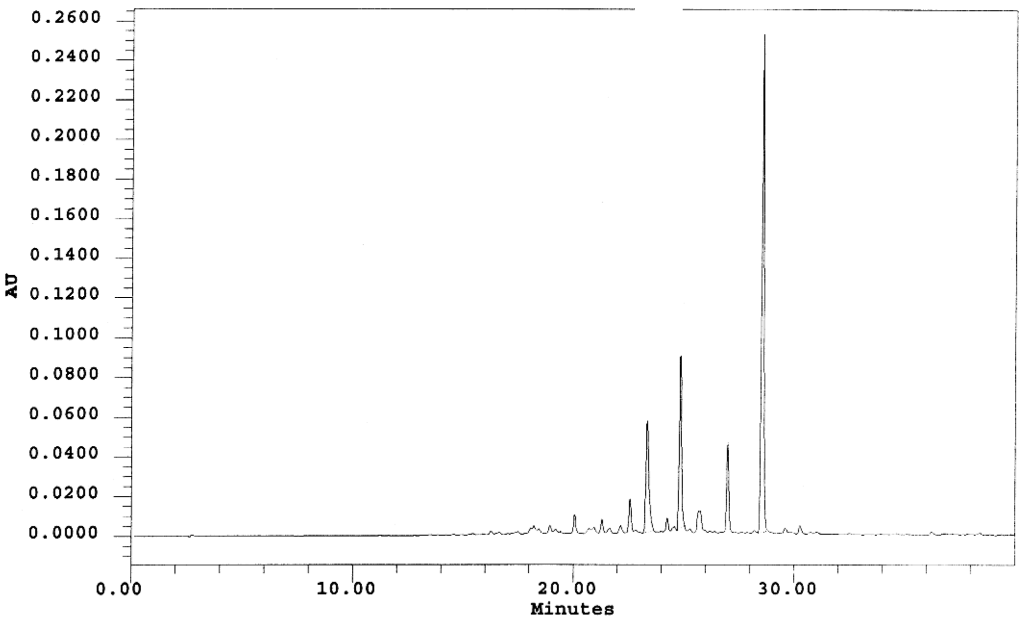
ret. time (min)	name
16.4	dihydroxyflavone (Mabry *et al.*, 1970)
22.9	flavonol
25.1	quercitrin
26.6	flavonoid
27.2	afzelin
28.8	flavonoid

Fig. II.9: *Cercis canadensis*: October leaves (detection at 350 nm).



ret. time (min)	name
24.9	flavone
25.7	flavone
27.0	flavone
28.6	flavone

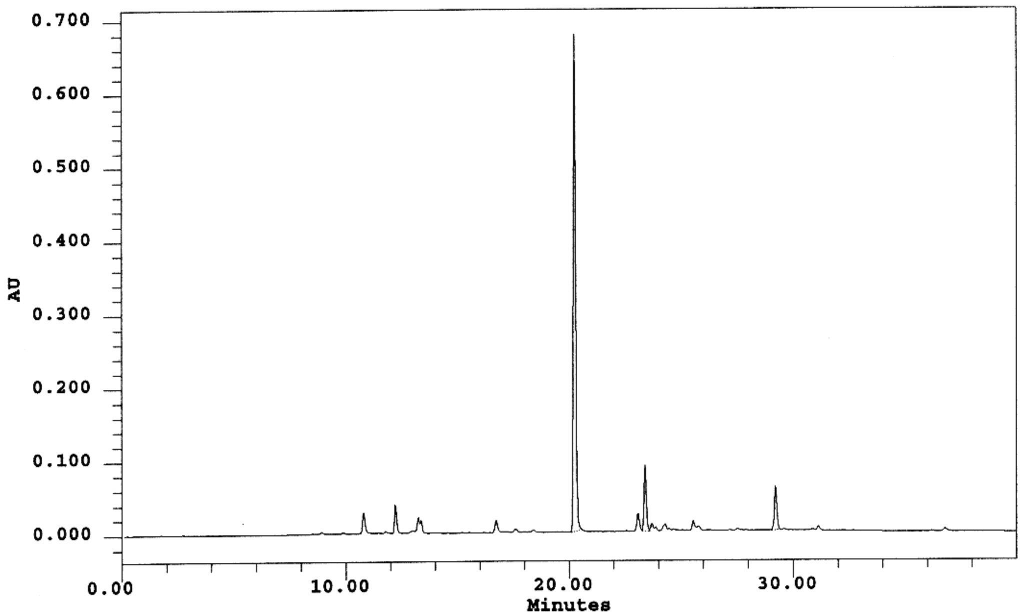
Fig. II.10: *Robinia pseudoacacia*: May leaves (detection at 350 nm).



ret. time (min)	name
23.4	luteolin derivative
24.9	flavone
27.0	flavone
28.6	flavone

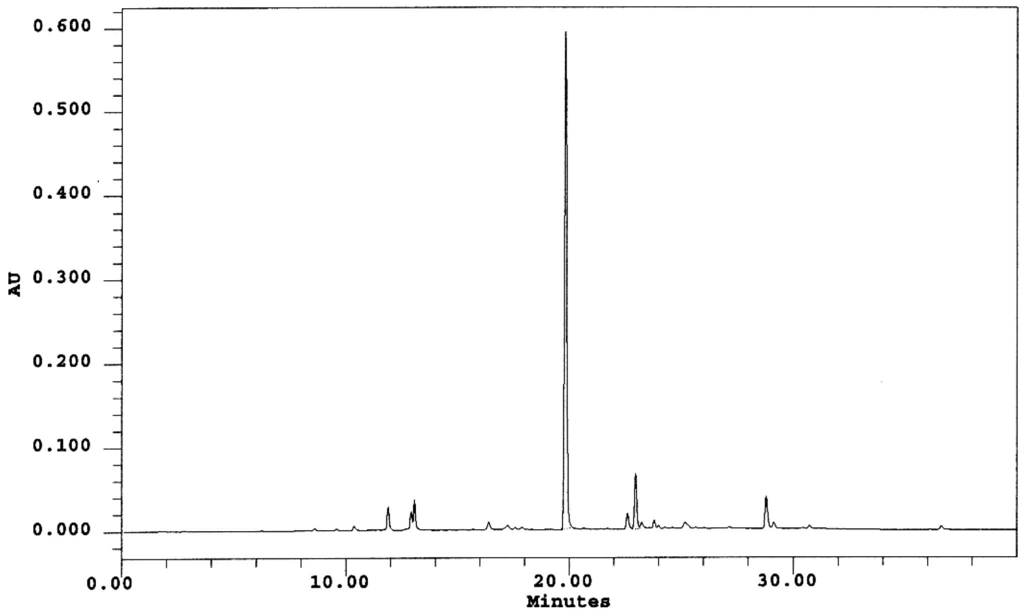
Fig. II.11: *Robinia pseudoacacia*: October leaves (detection at 350 nm).

Based on assignments from Mabry *et al.* (1970), the most common aglycone skeleton in *R. pseudoacacia* flavones (Fig. II.10 and Fig. II.11) is the 4’,5,7-trihydroxyflavone, which may differently be derived.



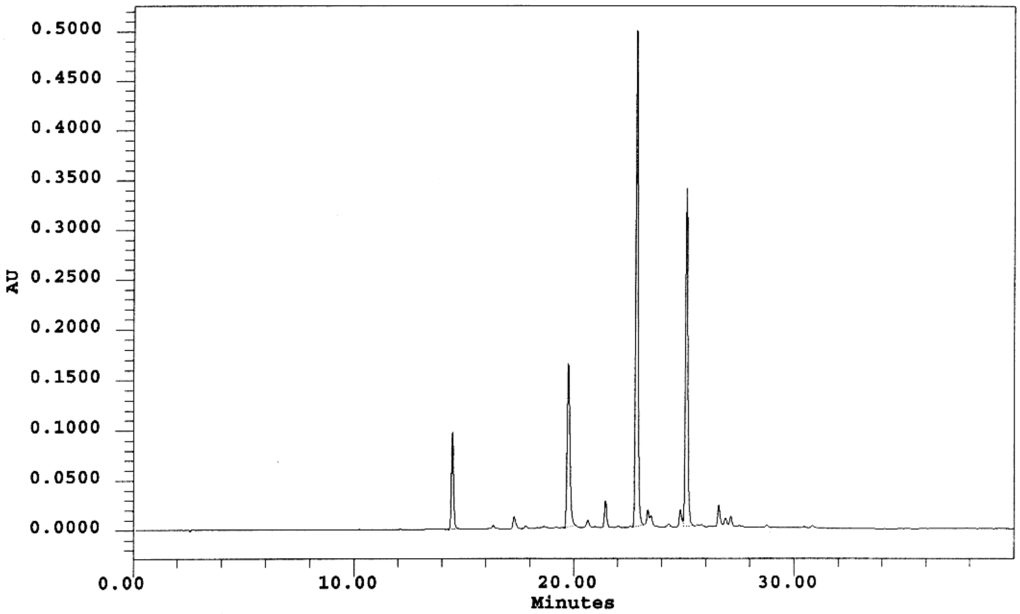
ret. time (min)	name
20.3	gallic acid derivative

Fig. II.12: *Acer rubrum*: May leaves (detection at 270 nm).



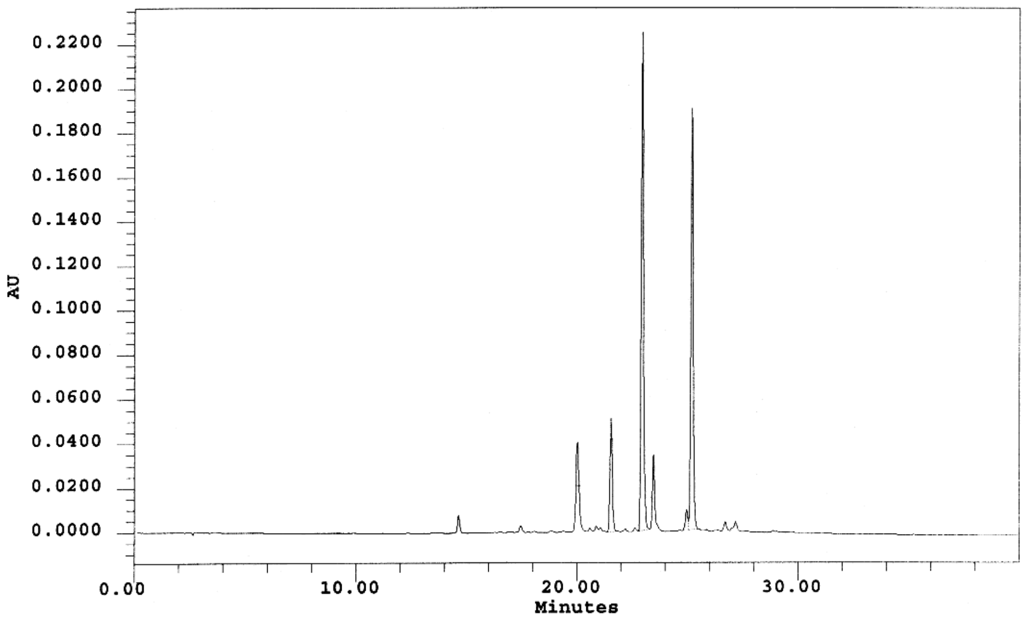
ret. time (min)	name
19.8	gallic acid derivative

Fig. II.13: *Acer rubrum*: October leaves (detection at 270 nm).



ret. time (min)	name
14.5	dihydroxyflavone (Mabry *et al.*, 1970)
19.8	flavonoid (tentatively identified as auronol)
22.9	flavonol
25.1	quercitrin

Fig. II.14: *Liquidambar styraciflua*: May leaves (detection at 350 nm).



ret. time (min)	name
19.9	flavonoid (tentatively identified as auronol)
21.5	flavonol
22.9	flavonol
23.4	quercetin derivative
25.1	quercitrin

Fig. II.15: *Liquidambar styraciflua*: October leaves (detection at 350 nm).

II.5 Investigation and characterisation of *A. julibrissin* leaf mucilage

5.1 Linkage analyses of the major monomer monosaccharide units by means of methylation analyses and subsequent GC-(EI)MS detection

Methylation analysis and subsequent detection by means of GC-MS analyses revealed the occurrence of 1,2- and 1,2,4-linked rhamnose as well as 1,4-linked galactose and 1,4-linked galacturonic acid units, respectively. Under EI conditions no intact monosaccharide units appeared as a molecule radical cation ($[M]^{+\bullet}$). In the following, only diagnostic fragments are depicted.
The molecular ions fragmented at their predestined positions between two neighbouring methoxy groups. The next preferred position for an intramolecular cleavage is generally between a neighbouring methoxy and acetoxy group, followed by two neighboured acetoxy groups.

GC-(EI)-MS of 1,2-linked rhamnose

After methylation analysis (C.V.2.2) the 1,2-linked rhamnose appeared as 1,2,5-tri-O-acetyl-3,4-di-O-methyl-rhamnitol with a molecular weight of 320 Da (its corresponding molecule radical cation was not detected in the spectrum). The retention time in the (gas) chromatogram was 12:12 min.

m/z 320	$[M]^{+\bullet}$	:	n.d. (not detected)
m/z 189	(39 %)	:	[fragment C1-C3]$^+$
m/z 131	(64 %)	:	[fragment C4-C6]$^+$
m/z 129	(100 %)	:	$[189 - CH_3COOH]^+$
m/z 99	(25 %)	:	$[131 - CH_3OH]^+$
m/z 89	(86 %)	:	[131 - ketene]$^+$
m/z 43	(56 %)	:	$[CH_3CO]^+$

GC-(EI)MS of 1,2,4-linked rhamnose

After methylation analysis the 1,2,4-linked rhamnose appeared as 1,2,4,5-tetra-O-acetyl-3-O-methyl-rhamnitol with a molecular weight of 348 Da. The retention time was 13:14 min.

m/z 348	$[M]^{+\bullet}$	:	n.d.
m/z 203	(26 %)	:	[fragment C3-C6]$^+$
m/z 189	(42 %)	:	[fragment C1-C3]$^+$
m/z 159	(5 %)	:	[fragment C4-C6]$^+$
m/z 143	(69 %)	:	$[203 - CH_3COOH]^+$
m/z 129	(100 %)	:	$[189 - CH_3COOH]^+$
m/z 43	(50 %)	:	$[CH_3CO]^+$

After the procedure of methylation analysis, only the hydroxyl group in position 3 was methylated, all other hydroxyl functions were acetylated. Therefore, the preferred sites for an intramolecular cleavage under EI-conditions are between C2 and C3, and between C3 and C4, respectively.

GC-(EI)MS of 1,4-linked galactose

After methylation analysis the 1,4-linked galactose appeared as 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactitol with a molecular weight of 350 Da. The retention time was 13:42 min.

m/z 350	$[M]^{+\bullet}$	:	n.d.
m/z 233	(43 %)	:	[fragment C3-C6]$^+$
m/z 173	(49 %)	:	[233 - CH_3COOH]$^+$
m/z 131	(26 %)	:	[173 - ketene]$^+$
m/z 117	(52 %)	:	[fragment C1-C2]$^+$
m/z 113	(44 %)	:	[173 - CH_3COOH]$^+$
m/z 99	(81 %)	:	[131 – CH_3OH]$^+$
m/z 43	(100 %)	:	[CH_3CO]$^+$

GC-(EI)MS of 1,4-linked galacturonic acid

After methylation analysis the 1,4-linked galacturonic acid appeared as 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-galactitol-6-d_2 with a molecular weight of 380 Da. The retention time was 14:57 min.

m/z 380	$[M]^{+\bullet}$	:	n.d.
m/z 263	(15 %)	:	[fragment C3-C6]$^+$
m/z 203	(27 %)	:	[263 - CH_3COOH]$^+$
m/z 161	(16 %)	:	[203 - ketene]$^+$ or fragment C1-C3
m/z 129	(74 %)	:	[161 - CH_3OH]$^+$
m/z 117	(51 %)	:	[fragment C1-C2]$^+$
m/z 87	(73 %)	:	[129 - ketene]$^+$
m/z 43	(100 %)	:	[CH_3CO]$^+$

5.2 Sequential analyses of oligosaccharide units by means of GC-MS (including MS/MS experiments) and ESI-MS/MS after partial hydrolyses

Partial hydrolysis with TFA at three different concentrations (0.1 N, 1 N, 4 N TFA, see C.V.2.2) yielded oligosaccharide units (with best results achieved by 4 N TFA), which were reduced with $NaBD_4$ and permethylated. Hence, oligosaccharide-alditol-1-*d* units were obtained, which were directly analysed by GC-MS (including MS/MS) and ESI-MS/MS spectrometry.

In the ESI-MS, especially the positive mode yielded valuable information.

The compositional and linkage analyses were used as a basis to assign the respective monosaccharide units to the monomers of the oligosaccharides investigated. Therefore, HexA was assigned to GalA (galacturonic acid), Hex to Gal (galactose), and dHex to Rha (rhamnose). Under these conditions alditol-1-*d* moieties constituted the reducing sugar moieties, respectively.

GC-(EI)-MS experiments

After hydrolysis of an aliquot of purified *A. julibrissin* leaf mucilage, four units of oligosaccharides were detected during the GC run (mainly diagnostic fragments are depicted here):

- HexA-dHex-ol-1-*d* (GalA→ Rha-ol-1-*d*); retention time: 18:11 min.;
- HexA-Hex-ol-1-*d* (GalA→ Gal-ol-1-*d*); retention time: 19:06 min.;
- a linear tetrasaccharide; retention time: 28:14 min., and
- a branched tetrasaccharide; retention time: 29:37 min. The latter substance only appeared in trace amounts in the GC spectrum.

Sequences were corroborated and verified by ESI-MS/MS experiments (see below).

EI-MS of HexA-dHex-ol-1-*d* (GalA 1→ 4 Rha-ol-1-*d*)

m/z 233	(61%)	:	[HexA moiety]$^+$ (terminal HexA)
m/z 206	(100 %)	:	[dHex-ol-1-*d* moiety]$^+$ (initial dHex)
m/z 201	(67 %)	:	[HexA moiety - CH_3OH]$^+$
m/z 174	(12 %)	:	[dHex-ol-1-*d* moiety - CH_3OH]$^+$
m/z 169	(19 %)	:	[HexA moiety - 2 CH_3OH]$^+$
m/z 142	(59 %)	:	[dHex-ol-1-*d* moiety - 2 CH_3OH]$^+$
m/z 134	(12 %)	:	[fragment C1-C3]$^+$ of dHex moiety
m/z 101	(29 %)	:	presumably inner-ring fragment
m/z 75	(24 %)	:	
m/z 59	(20 %)	:	[$COOCH_3$]$^+$ or [$CH(CH_3)OCH_3$]$^+$

Focus on higher masses; m/z 289 is base peak

m/z 289	(100 %)	:	[455 - 134 - CH_3OH]$^+$
m/z 257	(98 %)	:	[455 - 134 - 2 CH_3OH]$^+$

As previously mentioned, masses of the deoxyhexose moiety are assigned to rhamnose, while masses of the hexuronic acid moiety are assigned to galacturonic acid, which is based on the compositional analysis. Fragment m/z 134 of the rhamnose moiety indicates a substitution in 4 position. Hence, the disaccharide unit is concluded to be GalA 1→ 4 Rha-ol-1-*d* (MW 455). However, this finding is not contradictory to the results of the methylation analyses, where especially 1,2- and to lesser extent 1,2,4-linked rhamnose has been found. Under reaction conditions of the partial hydrolysis, the obviously weaker 2-linkage of the rhamnose is completely cleaved before methylation while the more stable 4-linkage of the rhamnose moiety remained largely unaffected. So, it can be assumed that the GalA 1→ 4 Rha unit originates from a branched 1,2,4-linked rhamnose moiety, that was originally further substituted in 2-position.

In the higher mass range, the following weak but important diagnostic fragments could be detected:

m/z 455	(< 1 %)	:	[M]$^{+\bullet}$
m/z 396	(3 %)	:	[M - 59]$^+$ with 59 mass units either due to $COOCH_3^\bullet$ or due to $CH(CH_3)OCH_3^\bullet$
m/z 377	(2 %)	:	[M - $CHDOCH_3$ - CH_3OH]$^+$
m/z 364	(15 %)	:	[396 - CH_3OH]$^+$
m/z 352	(14 %)	:	
m/z 345	(5 %)	:	[377 - CH_3OH]$^+$
m/z 333	(19 %)	:	[M - C1-C2 fragment of dHex-ol-1-*d* moiety - CH_3OH]$^+$. This excludes substitution at position 2 of the rhamnose residue.
m/z 320	(81 %)	:	[352 - CH_3OH]$^+$
m/z 319	(100 %)	:	[352 - CH_3OD]$^+$

EI-MS of HexA-Hex-ol-1-*d* (GalA 1→ 4 Gal-ol-1-*d*)

m/z 236	(68 %)	:	[Hex-ol-1-*d* moiety]$^+$
m/z 233	(86 %)	:	[HexA moiety]$^+$
m/z 201	(100 %)	:	[HexA moiety - CH_3OH]$^+$
m/z 172	(50 %)	:	[236 - 2 CH_3OH]$^+$
m/z 141	(42 %)	:	[201 - CH_3COOH]$^+$
m/z 101	(47 %)	:	
m/z 75	(40 %)	:	
m/z 59	(10 %)	:	[$COOCH_3$]$^+$
m/z 45	(19 %)	:	[CH_2OCH_3]$^+$

Focus on higher masses; m/z 296 is base peak

m/z 485	(2 %):	:	[M]$^{+\bullet}$
m/z 426	(4 %)	:	[M - $COOCH_3$]$^+$
m/z 396	(13 %)	:	[M - fragment C5-C6 of Hex-ol-1-*d* moiety]$^+$
m/z 395	(29 %)	:	[M - fragment C1-C2 of Hex-ol-1-*d* moiety]$^+$
m/z 394	(9 %)	:	[426 - CH_3OH]$^+$
m/z 363	(13 %)	:	[395 - CH_3OH]$^+$
m/z 319	(5 %)	:	[M - fragment C1-C3 of Hex-ol-1-*d* moiety - CH_3OH]$^+$
m/z 296	(100 %)	:	

From the EI-MS data, the HexA-Hex-ol-1-*d* unit combined with the results from the compositional analysis is therefore concluded to be GalA 1→ 4 Gal-ol-1-*d* (MW 485). In agreement with the methylation analysis, the linkage in position 4 of the galactose moiety is corroborated by the fragmentation pattern of the disaccharide unit.

Monitoring the higher masses, the following fragmentation pattern could be found:

m/z 395	(100 %)	:	[M - C1-C2 fragment of Hex-ol-1-*d* moiety]$^+$
m/z 376	(20 %)	:	
m/z 363	(55 %)	:	[395 - CH_3OH]$^+$
m/z 335	(17 %)	:	
m/z 319	(29 %)	:	[M - C1-C3 fragment of Hex-ol-1-*d* moiety - CH_3OH]$^+$
m/z 307	(60 %)	:	[335 - CO]$^+$

EI-MS of a HexA-dHex- unit, with
HexA being terminal, and dHex fragmenting within the chain (not initially)

m/z 407	(<1 %)	:	[HexA-dHex]$^+$ with HexA being terminal
m/z 375	(6 %)	:	[HexA-dHex - CH_3OH]$^+$
m/z 233	(60%)	:	[HexA moiety]$^+$ (terminal HexA)
m/z 206	(100 %)	:	[dHex-ol-1-*d* moiety]$^+$
m/z 201	(58 %)	:	[HexA moiety - CH_3OH]$^+$
m/z 169	(16 %)	:	[HexA moiety - 2 CH_3OH]$^+$
m/z 142	(23 %)	:	
m/z 111	(16 %)	:	
m/z 75	(12 %)	:	
m/z 59	(6 %)	:	[$COOCH_3$]$^+$ or [$CH(CH_3)OCH_3$]$^+$

After monitoring higher masses, the following could be found:

m/z 407	(6 %)	:	[HexA-dHex]$^+$
m/z 375	(100 %)	:	[407 - CH_3OH]$^+$
m/z 343	(32 %)	:	[375 - CH_3OH]$^+$

Fragment m/z 407 indicates GalA → Rha as repeating unit.

EI-MS of HexA-dHex-HexA-dHex-ol-1-*d* as linear tetrasaccharide unit

The molecule radical cation [M]$^{+\bullet}$ with a mass of 831 Da was not detectable in the spectrum.

m/z 697	(8 %)	:	[M - C1-C3 fragment of dHex-ol-1-*d* moiety]$^+$
m/z 658	(100 %)	:	
m/z 657	(95 %)	:	[M - dHex moiety]$^{+\bullet}$ with dHex being within the chain, not initial
m/z 638	(50 %)	:	
m/z 625	(31 %)	:	[M - dHex-ol-1-*d* moiety]$^+$
m/z 623	(43 %)	:	
m/z 609	(20 %)	:	[HexA-dHex-HexA- moiety]$^+$
m/z 595	(20 %)	:	
m/z 593	(16 %)	:	[625 - CH_3OH]$^+$

The linear tetrasaccharide is concluded to be a GalA→ Rha→ GalA→ Rha-ol-1-*d* unit. Loss of 134 mass units from the molecule radical cation corresponding to fragment C1-C3 of the initial dHex-ol-1-*d* moiety indicates a 4-linkage of this rhamnose moiety. No 1,4-linkage was found for rhamnose during the methylation analysis, therefore the 2-linkage may have been cleaved before detection by mass spectrometry. However, as noted in the methylation analyses, the largest peak in the GC spectrum originated from 1,2-linked rhamnose, the rhamnose moiety within the chain is thought to be 1,2-linked. Hence, it is reasonable to conclude that the sequence consists of 1,4-linked galacturonic acid, 1,2-linked rhamnose and a branched rhamnose moiety as the initial saccharide unit.

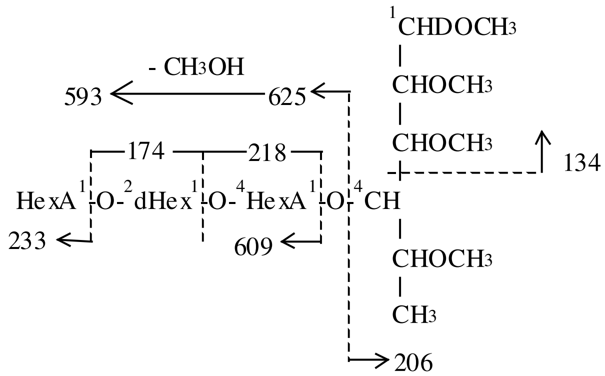


Fig. II.16: Fragmentation pattern of the linear tetrasaccharide unit HexA-dHex-HexA-dHex-ol-1-*d* under EI conditions.
Types of linkage within the chain are based on methylation analyses.

EI-MS of a HexA-Hex-(HexA)-dHex-ol-1-*d* as branched tetrasaccharide unit

The molecule radical cation $[M]^{+\bullet}$ exhibits a molecular weight of 877 Da and is not visible in the mass spectrum.

m/z 628	(4 %)	:	[HexA-Hex-dHex-ol-1-*d* unit]$^+$
m/z 424	(40 %)	:	[HexA-dHex-ol-1-*d*]$^+$ side chain
m/z 265	(12 %)	:	
m/z 233	(100 %)	:	[HexA moiety]$^+$ (terminal HexA)
m/z 201	(77 %)	:	[HexA moiety - CH_3OH]$^+$
m/z 160	(31 %)	:	
m/z 141	(14 %)	:	
m/z 111	(19 %)	:	
m/z 88	(24 %)	:	
m/z 44	(10 %)	:	$[CO_2]^{+\bullet}$

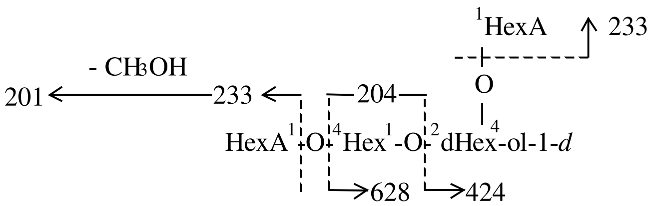


Fig. II.17: Fragmentation pattern of a branched tetrasaccharide unit under EI conditions.
Types of linkage are based on methylation analyses, and may be reversed at the branched site.

ESI-MS (positive mode)

The following masses occurred in the ESI-MS spectrum following hydrolysis with 4 N TFA, reduction with $NaBD_4$ and permethylation. MS/MS experiments were performed to corroborate the sequences assigned. All masses corresponded to the respective sodium adducts.

Detection of linear saccharide units

m/z 1654	(4 %)	:	[HexA-dHex-(HexA-dHex)$_2$-HexA-dHex-ol-1*d* + Na]$^+$; [M$_1$ + Na]$^+$
m/z 1262	(1 %)	:	[HexA-dHex-HexA-dHex-HexA-dHex-ol-1*d* + Na]$^+$; [M$_2$ + Na]$^+$
m/z 900	(1 %)	:	[HexA-Hex-HexA-dHex-ol-1-*d* + Na]$^+$; [M$_3$ + Na]$^+$
m/z 870	(8 %)	:	[HexA-dHex-HexA-dHex-ol-1-*d* + Na]$^+$; [M$_4$ + Na]$^+$
m/z 508	(100 %)	:	[HexA-Hex-ol-1-*d* + Na]$^+$; [M$_5$ + Na]$^+$
m/z 478	(66 %)	:	[HexA-dHex-ol-1-*d* + Na]$^+$; [M$_6$ + Na]$^+$

MS/MS experiments corroborated the occurrence of following sequences:

MS/MS of mass 1262 [M$_2$ + Na]$^+$

m/z 821	(18 %)	:	[HexA-dHex-HexA-dHex unit - H_2O + Na]$^+$
m/z 464	(100 %)	:	[HexA-dHex-ol-1*d* + Na]$^+$ with HexA not being terminal
m/z 429	(50 %)	:	[HexA-dHex - H_2O + Na]$^+$

M$_2$ is concluded to consist of the sequence **HexA-dHex-HexA-dHex-HexA-dHex-ol-1-*d*** as linear oligosaccharide unit (MW 1239). For masses generated in the ESI spectrum see Fig. II.18.

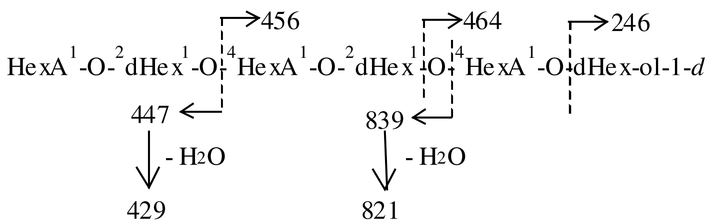


Fig. II.18: Fragmentation pattern of a linear hexasaccharide, indicates [HexA-dHex]$_n$ is a repeating basic structure. The sodium adduct of the molecule ion [M$_2$ + Na]$^+$, exhibits the mass m/z 1262. Likewise, all masses indicated, correspond to hydroxylated ions with sodium as adduct.
Types of linkage are based on methylation analyses.

MS/MS of mass 870 [M$_4$ + Na]$^+$

m/z 464	(100 %)	:	[HexA-dHex-ol-1*d* + Na]$^+$ with HexA not being terminal
m/z 429	(22 %)	:	[HexA-dHex - H_2O + Na]$^+$
m/z 397	(2 %)	:	
m/z 273	(2 %)	:	[HexA + Na]$^+$ HexA being terminal

Measurement with m/z 397 as base peak

m/z 397	(100 %)	:	
m/z 273	(70 %)	:	[HexA + Na]$^+$ HexA being terminal
m/z 246	(51 %)	:	[dHex-ol-1-*d* + Na]$^+$

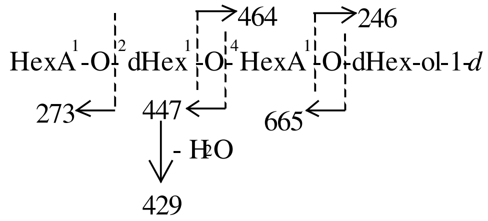


Fig. II.19: Fragmentation pattern of $[M_4 + Na]^+$, m/z 870. Masses depicted correspond to sodium adducts of hydroxylated ions.
Types of linkage are based on methylation analyses.

MS/MS of mass 508 $[M_5 + Na]^+$

m/z 276	(70 %)	:	[Hex-ol-1-*d* Na]$^+$
m/z 273	(39 %)	:	$[HexA + Na]^+$
m/z 228	(100 %)	:	246 - H_2O

MS/MS of mass 478 $[M_6 + Na]^+$

m/z 273	(100 %)	:	$[HexA + Na]^+$
m/z 255	(19 %)	:	$[HexA - H_2O + Na]^+$
m/z 246	(44 %)	:	[dHex-ol-1-*d* + Na]$^+$

HexA-Hex-(HexA)-dHex-ol-1-*d* as branched oligosaccharide unit

(compare Fig. II.17)

MS/MS of mass 900 $[M_3 + Na]^+$

m/z 668	(14 %)	:	$[M - HexA + Na]^+$
m/z 464	(100 %)	:	$[M - HexA\text{-}Hex + Na]^+$
m/z 273	(11 %)	:	$[HexA + Na]^+$

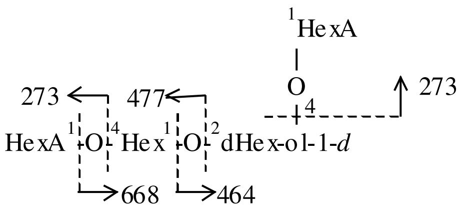


Fig. II.20: Fragmentation pattern of $[M_3 + Na]^+$, m/z 900, a branched tetrasaccharide. All masses indicated correspond to hydroxylated ions with sodium as adduct. Evidence for the occurrence of branching points.
Types of linkage are based on methylation analyses, and may be reversed at the branched site.

HexA-dHex as repeating unit

In agreement with the results from GC-MS, HexA-dHex (GalA→ Rha) was found to occur as repeating unit, which corresponds to a mass difference of 392 units, respectively:

m/z 1654 (2 %), 1262 (18 %), 870 (100 %)
m/z 1292 (2 %), 900 (32 %)

It should be further noted that the linkage between galacturonic acid and rhamnose (GalA-Rha) is purported to be more stable than rhamnose to galacturonic acid (Rha-GalA), as a reduced galacturonic acid has never been found as an initial sugar moiety of oligosaccharide units.

Curriculum vitae

Name: Annette Abhau
Date of birth: 07.03.1966
Native town: Mülheim a. d. Ruhr
Parents: Heinz Abhau
Johanna Abhau, nee Schmidt

School education

1972 - 1975	Städt. Gemeinschaftsgrundschule am Oemberg in Mülheim a. d. Ruhr (“Municipal Primary Interdenominational School Oemberg”)
1975 - 1985	Städt. Gymnasium Broich in Mülheim a. d. Ruhr (“Municipal Gymnasium Broich”)
02.05.1985	Abitur (school-leaving exam)

Practical training

09.04.1984 - 29.04.1984	Zoologischer Garten Hannover (Zoological Gardens Hanover)

University education

01.10.1985 - 31.03.1991	The study of pharmacy at the Heinrich-Heine-Universität Düsseldorf
01.09.1988	1. Staatsexamen (State examination)
04.09.1990	2. Staatsexamen (State examination)
15.01.1992	3. Staatsexamen (State examination)
05.02.1992	Receipt of the “Approbation als Apothekerin” (certificate as pharmacist)
06.04.1993	Receipt of the “Diplom als Diplompharmazeut” at the Martin-Luther-Universität Halle-Wittenberg

Practical year before being certified

01.10.1990 - 31.03.1991	St.-Florian Apotheke (Pharmacy) in Düsseldorf
01.04.1991 - 30.09.1991	Institut für Pharmazeutische Biologie der Philipps-Universität in Marburg / Lahn, supervised by Prof. Dr. J. Hölzl During this time doing a diploma with the subject: “Methode zur Bestimmung der Sesquiterpenlactone bei Reihenuntersuchungen von *Arnica montana* L.” (“Development of a method to determine sesquiterpenlactones in *Arnica montana* L. for series investigations”)

Practical training during the university studies at

10.03.1986 - 21.03.1986	Institut für Pflanzenbau und Pflanzenzüchtung der Justus-Liebig-Universität Giessen, Abteilung Arznei- und Gewürzpflanzenbau, Versuchsstation Rauischholzhausen, supervised by Prof. Dr. A. Vömel
04.08.1986 - 31.08.1986	the WALA-Heilmittel GmbH in Eckwälden / Bad Boll
24.05.1988 - 08.06.1988	the Hospital-Pharmacy of the Klinikum Kalkweg and Bertha-Krankenhaus Rheinhausen, Städtische Kliniken Duisburg (“Municipal Clinics of Duisburg”)

07.08.1989 - 19.08.1989	Institut für Pharmazeutische Biologie der Philipps-Universität in Marburg / Lahn, supervised by Prof. Dr. J. Hölzl

During the entire time of the studies I worked as temporary substitute at the Paracelsus-Apotheke (Pharmacy) in Duisburg.

Professional career

01.02.1992 - 30.09.1992	Bavier-Apotheke (Pharmacy) in Erkrath
16.01.1993 - 31.12.1993	Apotheke Kattenesch (Pharmacy) in Bremen
01.01.1994 - 01.07.1997	Schauburg-Apotheke (Pharmacy) in Delmenhorst
15.07.1997 - 31.03.1998	Apotheke Kattenesch (Pharmacy) in Bremen
01.04.1998 - 05.07.2002	Victoria-Apotheke (Pharmacy) in Bremen
since 08.1999	teaching pharmacology to midwives at the School Centre “Ev.-luth. Diakonissen Mutterhaus in Rotenburg / Wümme, Staatlich anerkannte Hebammenschule” (adaptation course for midwives from foreign countries).
since 01.02.2003	Schloss-Apotheke (Pharmacy) in Syke

Further education

17.01.1997 – 26.10.1997	Further education at the “Apothekerkammer Nordrhein” to a nutrition adviser
28.01.1998	Receipt of the certificate as nutrition adviser

PhD-project

01.01.1993 - 19.06.1993	Explored various subjects for a thesis with regular half-weekly stays at the Deutsches Primatenzentrum in Göttingen (German Primate Centre): Studying primatology and observing *Macaca silenus*.
19.06.1993 - 19.07.1993	Sojourn to Madagascar. During this time I visited the research camp of Joyce Powzyk at the Matadia National Parc. We observed free ranging *Indri indri* and *Propithecus diadema diadema*. In the field I also met Prof. Dr. K. Glander, who invited me to the Duke University Lemur Center to study the feeding behaviour of *Propithecus verreauxi*.
12.1993	Prof. Dr. T. Hartmann (Institut für Pharmazeutische Biologie der Technischen Universität Braunschweig), promised to supervise the pharmaceutical part of my project.
02.05.1994 - 24.05.1994	Observation of the feeding behaviour of *Propithecus verreauxi coquereli* at the Duke University Lemur Center, N.C., USA.
01.03.1997 - 15.06.1997	Chemical analyses at the Institut für Pharmazeutische Biologie der Technischen Universität Braunschweig, supervised by Prof. Dr. T. Hartmann, and at the Leibniz-Institut für Pflanzenbiochemie, IPB, Halle / Saale, supervised by Prof. Dr. D. Strack.
30.09.1997 - 09.10.1997	Follow-up study at the Duke University Lemur Center, N.C., USA.
23.09.1998 - 12.10.1998	Chemical analyses at the IPB, Halle / Saale.

Private career

21.06.1996	Marriage to Dipl. phys. Hermann Mathias Abhau, nee Schulz-Methke
05.07.1999	Birth of our daughter Anika Florence Abhau
2001	Building of our house and planting the garden
19.10.2003	Birth of our second daughter Melissa Katharina Abhau